Assessing stress physiology of cane toads in Florida: tradeoffs with dispersal

by

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A dissertation submitted to the Graduate Faculty of
Auburn University
in partial fulfillment of the
requirements for the Degree of
Doctor of Philosophy

Auburn, Alabama
May 1, 2021

Keywords: Eco-immunology; Invasion; endurance; LPS; Poison; *Rhinella marina*

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Abstract

Physiological tradeoffs occur in organisms coping with their environments, which are likely to increase as populations reach peripheries of established ranges, potentially due to stress. Invasive species offer opportunities to study tradeoffs that occur, such as immune responses and defensive behaviors. The cane toad (*Rhinella marina*) is a well-known invasive species. Populations near the expanding edge of the Australian invasion have altered immune responses, increased leg and poison (parotoid) gland size, and decreased likelihoods of fleeing when disturbed compared to toads from longer established core populations, although this has not been well-documented for Florida populations. The research of this dissertation focuses on evaluating how immune, defensive, and endurance levels of cane toads from a northern edge (New Port Richey (NPR)) and southern core (Miami) population in Florida differ due to location in Florida. Core population individuals injected with LPS showed greater metabolic increases compared to their baseline rates that were higher compared to those from the edge population, and core individuals had different circulating leukocyte profiles compared to saline-injected cane toads while edge individuals did not. There was also a significant interaction between location and time on circulating corticosterone (CORT) levels following injections with saline or LPS, with CORT decreasing more with time in core population toads. We found that residual body indices increased in individuals from higher latitude populations, and relative parotoid gland size increased with increasing toad size. There was no effect of latitude on the allometric relationship between gland size and toad size. We observed an increase in likelihood of secretion by cane toads in the field with increasing latitude. Edge population individuals were less willing to move and did not travel as far in the track as those from the core population; however, there was no effect of locality on the distances traveled by toads in a treadmill. Although lactate levels decreased with time, there was also no effect of locality on lactate metabolism. The CORT responses of these toads observed in the field and in lab indicates that differential stress responses contribute to the tradeoffs observed with immunity, behavior, and dispersal.

Acknowledgements

I would like to thank my dissertation committee members and my outside reader (Dr. Todd Steury) for their support, insights, and constructive critiques that substantially helped me to improve the quality of research found in this dissertation. I also would like to thank Vania Assis for her assistance with field and lab work, S. Hayworth and E. Yonan for their assistance with locomotion data collection, and the other undergraduates in the Mendonça lab who assisted with cane toad care and husbandry during my time at Auburn University. I would also like to acknowledge the CMB Peaks of Excellence fellowship offered by Auburn University for financial support both during my 1st year and during the summer of 2018.

I want to acknowledge my mother Peggy Gardner for all of her support during my childhood, the countless hours she spent with me at the local public library, and her keen interest in my continuing education. I also thank my late father Reverend Bobby Wayne Gardner for his unwavering support and belief in my potential, both academically and as a person. I thank him for working so hard to help me realize the opportunities I had and for all of his guidance and unconditional love. He is missed very dearly. Without the support of both of my parents, this dissertation would not be possible.

Finally, I would like to thank my love Jessica Ertel. She has been my best friend and emotional support during my years at Auburn University. Completing this dissertation would have been much more difficult without her love, belief in my abilities, and her ability to lighten my mood following many long days.

Cane toads used in the following experiments were collected under permits FFWC EXOT-17-34 and EXOT-19-58. Animal handling and protocols were performed under Auburn University IACUC PRNs 2017-3106 and 2020-3714.

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List of Abbreviations

NPR	New Port Richey
LPS	Lipopolysaccharide
BKA	Bacteria killing assay
GC	Glucocorticoid
CORT	Corticosterone
N:L	Neutrophil : Lymphocyte
$\dot{V}O_2$	Volume (ml) of oxygen consumed h^{-1}
MBG	Marinobufagenin
SVL	Snout-vent length
HPLC	High performance liquid chromatography
NSS	Normal saline solution
BI	Body condition index
PNMT	Phenylethanolamine-N-methyltransferase
RLL	Relative leg length

Chapter 1

Literature Review

Life History Theory

Life history theory attempts to explain variations among organismal traits (such as speed of development, immune investment, adult body size, reproductive investment, and mortality likelihood) in relation to the environments in which they live (Stearns 2000). Species that demonstrate patterns of having faster growth rates, lower immune investment, larger clutch sizes, and lower life spans are referred to as being "r-selected", while species showing opposing patterns are referred to as "k-selected" (Parry, 1981; Stearns, 1976). Even within species, individuals from differing populations with specific selection pressures may show r vs k selective traits (Reznick et al., 1990; Stearns, 2000), and eco-physiological studies have shown tradeoffs with investment into various physiological functions in organisms mediated by the differing environmental conditions in which they live (Norris & Evans, 2000; Sheldon & Verhulst, 1996). These tradeoffs occur from selection acting to maximize fitness within populations of these individuals (Tuomi et al., 1983). The abundant center and central-marginal hypotheses predict that populations in the center of their geographical range experience "optimal" conditions for survival, and as populations expand into novel habitats, conditions become less favorable to survival, leading to reduction of population sizes and elevation of stress in individuals near the expanding edge (Sagarin & Gaines, 2002; Eckert et al. 2008). These individuals may experience changes in morphological and behavioral characteristics as a result (Krause et al., 2016).

Invasion Biology

Invasions from introduced species offer unique opportunities for studying mechanisms of evolution and adaptation to new environments (Bock et al., 2015). In many cases, invasive species are detrimental to the habitats they are introduced to, as they affect diversity of native fauna by potentially spreading disease (Nally, et al., 2016), outcompeting and preying upon native species (Willson, 2017), and in the case of toxic invaders, poisoning native predators that have not evolved in sympatry with the invading species and or learned avoidance behaviors (Cabrera-Guzmán et al., 2012). Many invaders near expanding (edge) populations have increased growth rates (Edmonds et al., 2004) and higher reproductive output (Parker et al., 2013) compared with individuals from longer established (core) populations in the introduced range and compared with remaining individuals from the previous native range. Invasive species have also shown altered behaviors in introduced habitats compared to in their native range (Rehage & Sih, 2004; Sakai et al., 2001; Weis, 2010).

Studies have been performed to assess the invasive potential and success of introduced species, with many focusing on tradeoffs with immune responses and dispersal into their introduced habitats (Brown & Shine, 2014). Invaders are predicted, and in many studies are shown, to alter investment into immune responses (Lee et al., 2005; White & Perkins, 2012). Due to the high rates of activity and dispersal among many populations of these invaders (Brown and Shine 2014), it has been inferred that innate immune responses that are generally associated with elevated metabolic and inflammatory costs (Lee & Klasing, 2004) are downregulated in these organisms, or at least occur at lowered frequencies. The evolution of increased competitive ability (EICA) hypothesis predicts that a lowered innate immune response profile allows for higher ecological advantage compared to native species that must maintain these processes. The "escape" from the higher parasitic and pathogenic loads in the native ranges that occur upon introduction or dispersal into novel habitats (the "enemy-release" hypothesis (Lee and Klasing 2004)) then allows for the decreased investment into immune defenses by invaders to be less detrimental in these novel habitats compared with the native ranges. These hypotheses imply therefore that invasive organisms are able to allocate more energy to growth and reproduction, as well as further dispersal into the introduced range.

Glucocorticoids

Organisms experiencing novel or unpredictable conditions (such as introduced species invading novel territories) are likely to experience increases in glucocorticoid levels (Husak & Moore, 2008; Bonier et al. 2009), cholesterol-derived hormones secreted from adrenal (or interrenal glands in amphibians (Rollins-Smith, 2017)) following stimulation of the hypothalamic-pituitary axis from external or internal stimuli (Kloet et al., 2008). These hormones have multiple effects, among which include affecting metabolism through altering receptivity of tissues to insulin (Mcmahon et al., 1988), which serves to differentially allocate energy among physiological processes during times of hardship, as well as decreasing investment into reproduction (Schoech et al., 2009), and altering locomotive behavior (Kapoor et al., 2008). Exposure to chronic stressors may also lead to altered personality and behavioral differences among individuals experiencing them, which may lead to increased avoidance or fleeing tendencies, increased alertness, reductions in foraging, and reduced reproductive behaviors (Reeder & Kramer, 2005). Elevated GC levels in these organisms may also be responsible for the dampened immune responses observed from invasive species (Graham et al., 2012; Assis et al. 2020), as elevated GCs, such as corticosterone (CORT) in amphibians, modulate severity of immune responses, through such processes as interference with NF-kB activity required for expression of several cytokines (Padgett and Glaser, 2003), as well as inhibition of plasma complement activity (Packard & Weiler, 1983).

The effects caused by elevated glucocorticoid levels are likely a result of the organisms experiencing them attempting to maintain allostasis (Romero et al., 2009), and Gomes et al. (2012) predicted that populations experiencing higher baseline GC levels may show higher acclimation efficiency to increased or novel stressors in their respective environments. Genetic background (Evans et al., 2006), as well as maternal effects and early-life environment during development (Hayward & Wingfield, 2004) all lead to differences in stress responses that may influence reactivity to novel stressors (Koolhaas et al., 1999; Øverli et al., 2007).

Cane toads (*Rhinella marina*)

The cane toad, *Rhinella marina,* is a well-known toxic amphibian species that has become invasive throughout several parts of the world (Lever, 2001). Due to their voracious feeding responses, cane toads were introduced into Australia in the early to mid-1900's as a means of controlling sugar cane pests (Lever 2001; Phillips et al. 2006) and were released in Florida (Miami) in the United States prior to 1955 (Krakauer, 1968). These toads reproduce in large numbers, with females capable of laying up to 30,000 eggs per clutch in invaded areas (Hagman and Shine 2006). Cane toads from recently established (< 1yr) populations near expanding-edge populations in Australia have been observed having longer legs relative to their body size, as well as higher endurance compared to toads from longer established populations (Llewelyn et al., 2010), allowing for some individuals to reach dispersal rates of up to 55 km per year (Phillips et al. 2007). Much of the devastation to native Australia fauna and predators caused by the establishment and spread of cane toads has been due to their highly toxic secretions, common to bufonids and many other amphibians (Garg et al., 2007), that significantly impact the survivorship of native predators from the areas they invade (Shine, 2010). The parotoid macroglands that contain the cocktail of toxic compounds (bufadienolides) found in the cane toad secretions are innervated by the sympathetic nervous system (Hostetler & Cannon, 1974; Skoglund & Sjöberg, 1977), indicating that individuals that more readily secrete poison may be more sympathetically sensitive to disturbance or predation attempts.

Tradeoffs that have occurred from the introduction and subsequent spread of this toad species have been well-studied among populations in Australia (Shine 2010), among which include altered metabolic rates (Llewellyn et al., 2012), phagocytic ability (Brown and Shine 2014), inflammatory processes (Brown et al., 2011), circulating leukocyte profiles (Brown et al., 2015), and behaviors (Llewellyn et al. 2010) following immune challenges. In addition, morphological (Phillips et al., 2006), cold tolerance (McCann et al., 2014), and defensive behavioral changes (Hudson et al., 2017) have been reported.

A study by (Phillips et al. 2010) showed that populations in Australia closer to the invasion front have altered parasitic burdens, offering some support to the enemy release hypothesis, although individuals from populations near the expanding-edge populations in Australia have shown increased poison (parotoid) gland sizes relative to their body size and demonstrated higher likelihoods of secreting poison upon disturbance (Hudson et al. 2017). This allometric increase and secretion behavior may indicate potential predation threats as these toads invade novel areas, where predators have yet to learn avoidance of these toads (Cabrera-Guzmán et al., 2012). The study by Brown and Shine (2014) which showed differential stress and immune profiles of populations near the expanding edge, offers support to elevated GCs contributing to these differences.

Objectives, Hypotheses, and Predictions

Although many studies have been performed assessing invasive cane toad populations in Australia, few have been performed on those introduced in the 1950's (Lannoo, 2005) to the United States in Florida (Krakauer, 1968; Punzo & Lindstrom, 2001; Gardner et al., 2018; Mittan & Zamudio, 2019). Florida cane toads have spread northward along a latitudinal gradient, with the northern edge populations now being established for approximately 29 years (U.S. Geological Survey (2020), where they may experience harsher conditions compared to cane toads from the Australian populations that have spread across a longitudinal gradient. It is therefore likely that individuals from these populations may display similar trends in immunological responses and behaviors as those from Australian populations, perhaps even more so. However, as the edge populations in Florida have now been established for longer periods than the continually expanding Australian edge populations, it is likely that these individuals do not display the same endurance and leg morphology changes as Australian cane toads.

The objectives of the research in this dissertation were to: 1. assess immune responses of northern (edge) and southern (core) populations of invasive Florida cane toads, 2. assess morphological, behavioral, and physiological characteristics reflecting toxicity risk of *R. marina* from well-established sites in southern Florida and from sites stretching northward to the invasion front, and 3. evaluate how locomotive capability has been affected from establishment throughout the state, as well as to assess whether Florida cane toads follow the same locomotion-capability trends as expanding-edge individuals in the Australian invasion. As Australian cane toads have shown attenuated immune responses, increased parotoid gland morphology and secretion behaviors, and Florida cane toads have spread along a latitudinal gradient in Florida (which likely confers additional stress to the northern-established individuals) our hypotheses are that: 1. Immune responses will vary among cane toads based on population locality in Florida 2. Parotoid gland morphology and secretion likelihood will vary among cane toad population locality in Florida and 3. Endurance levels and leg morphology will be similar among population locality in Florida. Our predictions are that: 1. individuals from a population near the expanding edge in northern Florida will have attenuated immune responses compared with those from a longer established population from the southern “core” of the range, 2. individuals from northern populations will show increased relative parotoid gland sizes and secretion likelihood compared to southern cane toads, and 3. that individuals from northern populations will show similar endurance levels as conspecifics from southern Florida, differing from the patterns of Australian cane toads.

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Chapter 2

Innate immunity of Florida cane toads: how dispersal has affected physiological responses to LPS

(Formatted for the Journal of Comparative Physiology B)

The final publication is available at:
https://link.springer.com/article/10.1007/s00360-020-01272-7

Abstract

Physiological tradeoffs occur in organisms coping with their environments, which are likely to increase as populations reach peripheries of established ranges. Invasive species offer opportunities to study tradeoffs that occur, with many hypotheses focusing on how immune responses vary during dispersal. The cane toad (*Rhinella marina*) is a well-known invasive species. Populations near the expanding edge of the Australian invasion have altered immune responses compared to toads from longer established core populations, although this has not been well-documented for Florida populations. In this study cane toads from a northern edge (New Port Richey (NPR)) and southern core (Miami) population in Florida were collected and injected with lipopolysaccharide (LPS) to compare immune responses. Core population individuals injected with LPS showed greater metabolic increases compared to their baseline rates that were higher compared to those from the edge population. In addition, LPS-injected core individuals had different circulating leukocyte profiles compared to saline-injected cane toads while edge individuals did not. There was a significant interaction between plasma bacteria killing capability (BKA) and treatment, such that BKA decreased with time in saline compared to LPS-injected individuals, and saline-injected toads from the edge population had lower

BKA compared to LPS-injected edge toads at 20h post-injection. There was also a significant interaction between location and time on circulating corticosterone (CORT) levels following injections with saline or LPS, with CORT decreasing more with time in core population toads. The differential CORT response indicates that differential stress responses contribute to the tradeoffs observed with immunity and dispersal.

Introduction

Eco-physiological studies have shown tradeoffs of investment into various physiological functions in organisms (Sheldon and Verhulst 1996; Norris and Evans 2000), mediated by differing environmental conditions. These tradeoffs occur from selection acting to maximize fitness within populations (Tuomi et al. 1983). The *abundant center* and *central-marginal hypotheses* predict that populations in the center of their geographical range experience “optimal” conditions for survival, and as populations expand into novel habitats, conditions become less favorable. This may lead to reduction of population sizes and elevation of stress in individuals near the expanding edge (Sagarin and Gaines 2002; Eckert et al. 2008).

Individuals experiencing novel or unpredictable conditions are likely to experience glucocorticoid (GC) level increases (Husak and Moore 2008; Bonier et al. 2009). It has been predicted that populations experiencing higher baseline GC levels may show higher acclimation efficiency to increased or novel stressors in their respective environments (Gomes et al. 2012). Genetic background (Evans et al. 2006), as well as maternal effects and early-life environment during development (Hayward and Wingfield 2004; Kitaysky et al. 2005) all lead to differences in stress responses that may influence reactivity to novel stressors (Koolhaas et al. 1999; Øverli et al. 2007). In addition to metabolic, reproductive, and behavioral effects of elevated GCs, tradeoffs with immune responses also occur (Graham et al. 2012).

Many studies addressing the potential and success of introduced species in becoming invasive have focused on tradeoffs with immune responses and dispersal into novel habitats (Brown and Shine 2014). Invaders are predicted to lower investment into immune responses (Lee et al. 2005; White and Perkins 2012). Due to high rates of activity and dispersal among many populations of invasive species (Brown and Shine 2014), it has been inferred that innate immune responses generally associated with elevated metabolic and inflammatory costs (Lee and Klasing 2004) are downregulated in dispersing organisms. The *evolution of increased competitive ability* (EICA) *hypothesis* predicts that a lowered innate immune response profile allows for higher ecological advantage compared to native species that must maintain these processes. The "escape" from higher parasitic and pathogenic loads in native ranges that occur upon introduction or dispersal into novel habitats (the "*enemy-release*" *hypothesis* (Lee and Klasing 2004)) then allows for decreased immune investment to be less detrimental in these novel habitats compared with native ranges. These hypotheses therefore imply that invasive organisms are able to allocate more energy to growth and reproduction, as well as further dispersal into the introduced range. Elevated GC levels in these organisms may be responsible for the dampened immune responses observed, as elevated GCs, such as corticosterone (CORT) in amphibians, modulate severity of immune responses though such processes as interfering with NF-kB activity required for expression of several cytokines (Padgett and Glaser 2003), as well as inhibition of plasma complement activity (Packard and Weiler 1983).

Multiple studies have used injections with lipopolysaccharide (LPS) to stimulate the immune systems of organisms to measure innate immunity (Bertrand et al. 2006; Lee et al. 2006; Llewellyn et al. 2012; Goetz et al. 2017; Gardner et al. 2018). LPS interacts with toll-like receptors, which leads to increased expression of various cytokines, including IL1B, IL6, IL8, and TNFa (Martin et al. 2011; Gardner et al. 2018; Scalf et al.

2019). These have differing effects in the organisms expressing them, such as stimulating vasodilation and the release of adrenocorticotropic hormone from the HPA axis (Zou et al. 2000; Dinarello 2009; Andino et al. 2012; Varela et al. 2014), stimulating release of c-reactive proteins from hepatocytes (Castell et al. 1989), attracting neutrophils to sites of infection (Bickel 1993), and affecting oxygen transport (Nemeth et al. 2004), and metabolic rates (Tredget et al. 1988; Mahony et al. 1990; Van Der Poll et al. 1991; Stouthard et al. 1995). In addition, LPS activates complement activity (Morrison and Kline 1977) through various means (such as the classic pathway via interaction with C1 in the complement pathway through interaction with the lipid A component of LPS (Loos et al. 1974; Galanos et al. 1971) and through the alternate pathway (Gotze and Müller-Eberhard 1971; Dierich et al. 1973). Due to the diverse physiological effects that occur from LPS injections, many researchers in the field of eco-immunology have compared metabolic rates (Lee et al. 2005; Llewellyn et al. 2012; Goetz et al. 2017; Smith et al. 2017), neutrophil : lymphocyte ratios (Goessling et al. 2017; Selechnik et al. 2017), and plasma bacteria killing ability (BKA) (assessed by comparing growth of a known concentration of bacteria incubated with plasma from the organism of interest, compared with growth of a control suspension not incubated with plasma over time (Assis et al. 2013; Brown et al. 2015; Gervasi et al. 2014; Goessling et al. 2017; Goetz et al. 2017)) to assess energetic costs, changes in circulating leukocyte profiles, and complement activity in organisms mounting immune responses.

The cane toad, *Rhinella marina,* is an invasive species throughout several parts of the world (Lever 2001). Tradeoffs that have occurred from the introduction and subsequent spread of this toad have been well-studied among populations in Australia

(Shine 2010), including altered metabolic rates (Llewellyn et al. 2012), phagocytic ability (Brown and Shine 2014), inflammatory processes (Brown et al. 2011), circulating leukocyte profiles (Brown et al. 2015), and behaviors (Llewellyn et al. 2010) following immune challenges. Phillips et al. (2010) showed that populations closer to the invasion front along a longitudinal gradient in Australia have altered parasitic burdens, offering some support to the *enemy release hypothesis*, and Brown and Shine (2014) showed differential stress and immune profiles in populations near the expanding edge, offering support to elevated GCs contributing to these differences. Although many studies have been performed assessing invasive cane toad populations in Australia, few have been performed on those introduced in the 1950's to the United States in Miami-Dade county Florida (Krakauer 1968; Punzo and Lindstrom 2001; Gardner et al. 2018) which then expanded northward into more temperate areas (Mittan and Zamudio 2019), reaching Pasco county in 1991 (U.S. Geological Survey, 2020). As cooler environments present additional stress to ectotherms (Eikenaar et al. 2012; Dupoué et al. 2013; Trumbo et al. 2016), this northward expansion may compound the tradeoffs that have occurred from dispersal in the Florida cane toad invasion.

The objective of this research was to assess immune response differences between populations of invasive cane toads in Florida. Our hypothesis was that a population near the expanding edge in northern Florida should have attenuated immune responses compared with a longer established population from the southern "core" of the range. Our predictions were that cane toads from New Port Richey (NPR) (Pasco county, FL), a population near the expanding edge (first reported in 2007 (U.S. Geological Survey, 2020)), would show a lowered energetic investment into innate immune responses (assessed via metabolic rates), as well as lowered immune parameters such as circulating neutrophils (as higher neutrophils are often indicative of infection in amphibians (Brown et al. 2011; Peterson et al. 2013; Gervasi et al. 2014; Greenspan et al. 2017)) and plasma bacteria killing ability, all following an innate immune challenge with lipopolysaccharide (LPS), when compared with the Miami (Miami-Dade county, FL) cane toad population, (a southern “core” population). We predicted that these differences should be modulated by differential stress profiles (induced from northward dispersal), assessed via circulating CORT levels.

Materials and Methods:

Animal collection and husbandry

Cane toads (mass ~ 100g) were collected on 17-June-2018 and 18-June-2018 from Miami (25.7907° N, 80.1300° W) (n = 34) and New Port Richey (28.2442° N, 82.7193° W) (n = 33), respectively, representing the southern introduced core population and a northern edge population along the invasion front in Florida, and transported them to Auburn AL. The toads were placed individually into large plastic bins (60.96 x 46.99 x 40 cm) lined with paper towels and containing a large water bowl, and kept at a temperature of 25°C (± 2 °C). The bins were covered with mesh to allow airflow, and the toads were fed twice weekly for 2 weeks with 10 – 15 adult crickets, for 1 month until 4 days prior to metabolic trials.

Metabolic trials

Metabolic measurements were obtained following an immune stimulus on a subset of the toads (n = 16, 15 core and edge populations, respectively) in subsets of up to 6 toads at a time. Mass and the sex of individual toads were recorded prior to each trial. Toads were placed into 300 ml glass mason jars that served as metabolic chambers. The jars were covered with mesh, and lined with moist paper towels to prevent desiccation. The jars containing the toads were placed at 32^{o}C for a 3h acclimation period, a temperature at which this species has been shown to mount high responses to LPS injection (Sherman and Stephens 1998; Llewellyn et al. 2012). Following this acclimation, the jars were sealed with lids fitted with two, two-way stopcocks and tubing to allow air flow to be directed to and from the jars. Stop-flow respirometry was then performed on the toads for 20h, with measurements beginning at 7 p.m. (DST), 1h following each of the jars being sealed. Each jar was flushed with dry, CO_2-free air for 9 min (500 ml min^{-1}) using a Sable Systems (Sable Systems International, North Las Vegas, NV, USA) mass flow system (MFS2). Airflow was then redirected to a new jar using a Sable Systems V3 multiplexer, and the previous jar was sealed for 1h. Excurrent airflow was scrubbed of CO_2 and water vapor using soda lime and drierite, respectively, and O_2 readings were analyzed using a Sable Systems Oxilla II dual absolute differential O_2 analyzer. The toads were removed from the jars following the 20th cycle (20h). These initial measures provided baseline metabolic rates for each toad. After the baseline metabolic runs, the toads were returned to their bins to rest for a 24h period. Following this rest period, the toads were placed back into the jars at 32^{o}C for 3h. The toads were then removed temporarily from the jars, and immediately given an injection into the dorsal lymph-sac with either sterile amphibian saline (saline with 30% more water), or 20 µg g body mass^{-1} LPS (strain 0111:B4) (Millipore Sigma, St. Louis, MO) prepared in sterile amphibian saline at a concentration of 5 mg ml^{-1}. Following injections, each toad was immediately placed back inside the sealed jars, and metabolic rates were obtained for 20h, with measurements beginning 1h following injections. The toads were again removed following the 20th cycle (20h), and blood samples were collected (approximately 0.1-0.2 ml) via cardiac puncture. An additional subset of the toads (18 per population) was treated in the same manner as the toads from the metabolic trials, and injected with either LPS (n = 12) or saline (n = 6) 24 h following an initial 24h acclimation period to the jars; however, these toads were allowed to react to the treatments for 2h instead of 20h, consistent with a study by Gardner et al. (2018) in which RNA sequences and levels of gene expression of proinflammatory cytokines were obtained from cane toads responding to an LPS treatment for 2h. The toads were then removed from the jars and blood samples were collected via cardiac puncture. This was done to obtain blood parameters of cane toads during their early response and compare with circulating CORT, neutrophil : lymphocyte (N:L) ratios, and BKA following 20h of response to the treatments. A drop of the whole blood from blood samples was used to assess circulating N:L ratios, and plasma from centrifuged blood (4 min at 3000 rpm) was used to assess BKA and CORT levels for all toads during both time points. Metabolic data was obtained using Sable Systems ExpeData version 1.9.14 software.

N:L ratios

A drop of blood collected from each cane toad (from 2h and 20h subsets) was streaked across a frosted microscope slide. Slides were allowed to dry for 30 min, then covered with methanol and dried overnight. The following day, the slides were stained with Protocol Hema 3 solutions I and II (Fisher Scientific Company, Kalamazoo, MI) for 30s each, then rinsed gently and allowed to dry. Stained leukocytes and lymphocytes were recorded for each slide at 100x magnification, and N:L ratios were recorded.

BKA

BKA was evaluated from cane toads (2h and 20h post-injection with either saline or LPS) using the methods of Assis et al. (2013). Pellets of non-pathogenic *Escherichia coli* were suspended in 1 ml sterile PBS, and an aliquot (100 μL) was transferred to 5 ml TSB, and incubated overnight at 31°C. Bacterial concentration was measured at 600nm using a BioTek PowerWave XS spectrophotometer the following day. Using results from this initial reading, an aliquot of this bacteria solution was diluted in sterile amphibian ringer solution to obtain a working solution concentration of 10^6 bacteria ml^{-1}. Cane toad plasma samples from both treatments that were diluted (1: 20) in Ringer's solution (10 μL plasma: 190 μL Ringer), were mixed with 10 μL of working solution (~10^4 microorganisms), and incubated for 60 min at 23°C (± 1°C). A positive control consisting of 10 μL working solution in 200 μL of Ringer's solution (with no toad plasma), and a negative control (containing only Ringer's solution, 210 μl) were incubated under the same conditions. Following incubation, aliquots of TSB (500 μl) were added to each sample. Samples were vortexed thoroughly, and 300 μL of each were added (in duplicate) to a 96 well microplate. The microplate was incubated at 31°C for 2h, and the optical density of the samples was then measured hourly at 600nm, for a total of four readings. BKA was calculated according to the formula:

$$\left(1-\left(\frac{sample\ optical\ density}{positive\ control\ optical\ density}\right)\right)x\ 100$$

which represents the percent of killed microorganisms in the samples compared to the positive control. BKA was evaluated following the first reading marking the exponential growth phase of the positive controls.

Circulating CORT

Plasma samples were extracted according to the methods of Mendonça et al. (1996). Extracted samples were resuspended in enzyme immunoassay (EIA) buffer, and CORT concentrations were measured using ELISA kits (supplier no. 501320-96) (Cayman Chemical Company, Ann Arbor, MI). Intra-assay variation for CORT was 23.72 ($\pm$ 2.92) % and inter-assay variation, estimated using the average of four intermediate values from the standard curve (recommended by the kit instructions), was 25.98 ($\pm$ 4.64) %. Sensitivity of the assays were 29.52 ($\pm$ 4.45) ng ml^{-1}.

Statistical Analyses

A repeated measures model (using the lme function from the nlme package in R, version 3.4.3) was used to assess the effects of toad size (average mass) on baseline metabolic rates of cane toads obtained prior to the toads receiving amphibian saline or LPS, using location (edge vs core) and sex as factors and time and log-transformed toad mass as covariates, and log-transformed ml of O_2 consumed h^{-1} ($\dot{V}O_2$) as the dependent variable. Baseline metabolic rates decreased with time ($t_{563} = -7.35$, $P < 0.01$), although there was no significant effect of location ($t_{26} = 0.57$, $P = 0.58$) or sex ($t_{26} = 0.62$, $P = 0.54$). Log-transformed toad masses had a significant effect on baseline log-transformed metabolic rates ($t_{26} = 4.09$, $P < 0.01$), the slope of which was 0.826. We used this value as the mass-scaling coefficient for the rates of the toads responding to the saline or LPS treatments (Lighton 2018). After using the mass-scaling coefficient to convert all metabolic rates of the toads, a repeated measures model using time as a covariate and sex as a factor was used to assess metabolic differences among the treatments (saline and LPS-injections, along with baseline metabolic rates for each treatment within each population), using individual toad ID as the random effect. An initial model using toad sex and location as factors and time as a covariate to assess CORT differences between the two populations responding to the treatments (using the lm function in R) showed that there was a significant interaction between time and location ($t_{60} = -2.17$, $P = 0.03$), such that CORT in cane toads from the core population (n = 17, 16 (2h and 20h, respectively)) decreased by 1.41 ($\pm$ 0.66) ng ml^{-1} h^{-1} (regardless of treatment) compared with edge population toads (n = 18,15 (2h and 20h, respectively)). Therefore, the differences among the treatment groups for these two time points were analyzed separately. A model using toad sex as a factor and CORT as a covariate was used to assess differences among treatments in NL ratios among the treatments. For assessing BKA differences, a significant interaction was found between treatment and time ($t_{50} = -2.45$, $P = 0.01$) for

BKA levels in cane toads responding to the treatments, such that BKA in saline-injected cane toads (n = 10, 10 (2 and 20h, respectively)) decreased by 1.8 (± 0.7) % h^{-1} compared to LPS-injected cane toads (n = 21, 16 (2 and 20h, respectively)), so differences among treatments for each time point were analyzed separately. Resulting *P* values for comparisons among metabolic rates, CORT, NL ratios, and BKA were adjusted using Bonferroni correction in R. All figures were made using R version 3.4.3 and Inkscape version 0.92.3.

Results:

Mass-Independent metabolic rates

Cane toad mass-independent metabolic rates (n = 31) decreased by 0.003 (± 0.0002) $\dot{V}$O2 $(g)^{-0.826}$ h^{-1} ($t_{1175} = -10.28$, $P < 0.01$) (values represent mean (±) standard error), but were not affected by sex (n = 10, 21 (female and male toads, respectively) ($t_{29} = -0.35$, $P = 1$). There were no differences in mass-independent metabolic rates between the saline treatment groups (n = 6,5 (edge and core cane toads, respectively) ($t_{1175} = -1.82$, $P = 1$), between LPS treatment groups (n = 9, 11 (edge and core, respectively) ($t_{1175} = -0.879$, $P = 1$), or between LPS and saline treatment groups within each population (n = 9,6 (LPS and saline for the edge population ($t_{1175} = 1.89$, $P = 1$), and n = 11, 5 (LPS and saline for the core population ($t_{1175} = 0.51$, $P = 1$), respectively). Compared to baseline rates within each treatment group, only cane toads from the core population responding to the LPS treatment had elevated mass-independent metabolic rates (0.022 (± 0.005) $\dot{V}$O2 $(g)^{-0.826}$ h^{-1} ($t_{1175} = 4.68$, $P < 0.01$) ($P = 1$ for all other treatment – baseline comparisons).

The most pronounced period of increased rates for core toads injected with LPS was a 6h period from 9-14h post-injection, during which the average percent increase compares to baseline rates was 36.62 (± 7.21) % (Fig. 2.1) (for full list of means and standard errors, as well as data ranges and medians for metabolic as well as NL ratio, BKA, and CORT data, see Table 2.1). The rate increase for core toads responding to LPS during this period was 26.43 (± 9.21) % higher than the percent change in LPS-injected toads from the edge population (t_{25} = 2.87, P = 0.03), and 32.39 (± 11.04) % higher than the change in saline-injected toads from the core population (t_{25} = 2.93, P = 0.03) (for full 20 hour period, see Fig. 2.2).

Table 2.1 Data Ranges, medians, means, and standard errors of metabolic, neutrophil : lymphocyte (NL) ratios, plasma bacteria killing ability (BKA), and corticosterone (CORT) data obtained from cane toads from a northern edge and a southern core population in Florida.

Variable Measured	Treatment	Number of individuals	Number of measurements	range	median	mean	st error
Metabolic Rate (full 20hr)	saline – core baseline	4	100	0.118 – 0.440	0.199	0.205	0.006
	LPS – core baseline	11	219	0.038 – 0.730	0.18	0.198	0.006
	saline – edge baseline	5	99	0.112 – 0.321	0.158	0.168	0.004
	LPS – edge baseline	9	176	0.094 – 0.418	0.18	0.198	0.005
	saline – core	5	100	0.117 – 0.326	0.21	0.211	0.004
	LPS – core	11	220	0.125 – 0.447	0.211	0.22	0.003
	saline – edge	6	120	0.083 – 0.348	0.159	0.174	0.005
	LPS – edge	9	180	0.137 – 0.500	0.205	0.207	0.003
Percent increase (9 – 14hr)	saline – core	5	30	-45.39 – 50.77	4.56419	1.98	3.58
		11	65	-35.75 – 433.78	26.5186	36.62	7.21
		5	30	-30.17 – 55.56	-11.34	-1.49	4.2
		9	54	-44.89 – 43.20	9.33752	11.6	4.71

NL ratios (2 hr)	saline – core	4	4	0.132 – 0.233	0.213	0.198	0.023
	LPS – core	10	10	0.247 – 0.906	0.873	0.904	0.151
	saline – edge	5	5	0.082 – 0.424	0.197	0.251	0.062
	LPS – edge	12	12	0.111 – 1.061	0.709	0.631	0.092
NL ratios (20hr)	saline – core	5	5	0.106 – 0.545	0.213	0.377	0.074
	LPS – core	9	9	0.526 – 2.063	1.205	1.16	0.167
	saline – edge	5	5	0.075 – 1.00	0.225	0.346	0.171
	LPS – edge	7	7	0.338 – 1.848	0.661	0.748	0.199
BKA (2hr)	saline – core	4	4	69.05 – 95.24	85.71	83.93	5.44
	LPS – core	9	9	19.05 – 100.00	83.33	74.6	8.66
	saline – edge	6	6	7.14 – 100.00	88.1	75.4	14.27
	LPS – edge	12	12	2.38 – 97.62	88.1	67.86	9.78
BKA (20hr)	saline – core	5	5	51.85 – 81.48	70.37	70.37	5.49
	LPS – core	8	8	66.67 – 92.59	88.89	87.04	3.05
	saline – edge	5	5	3.70 – 70.37	51.85	48.15	11.89
	LPS – edge	8	8	62.96 – 88.89	77.78	75.93	2.8
CORT (2hr)	saline – core	6	6	6.11 – 66.14	19.03	27.72	9.51
	LPS – core	11	11	5.66 – 149.70	34.76	46.19	11.97
	saline – edge	6	6	4.19 – 25.08	12.38	12.74	3.14
	LPS – edge	12	12	8.09 – 76.74	16.72	27.63	7.02

CORT (20hr)	saline – core	6	6	7.00 – 26.76	12.2	14.69	3.54
	LPS – core	10	10	7.29 – 75.22	18.67	24.8	6.65
	saline – edge	6	6	8.54 – 38.40	21.68	24.13	4.55
	LPS – edge	9	9	6.59 – 70.82	32.5	33.46	6.61

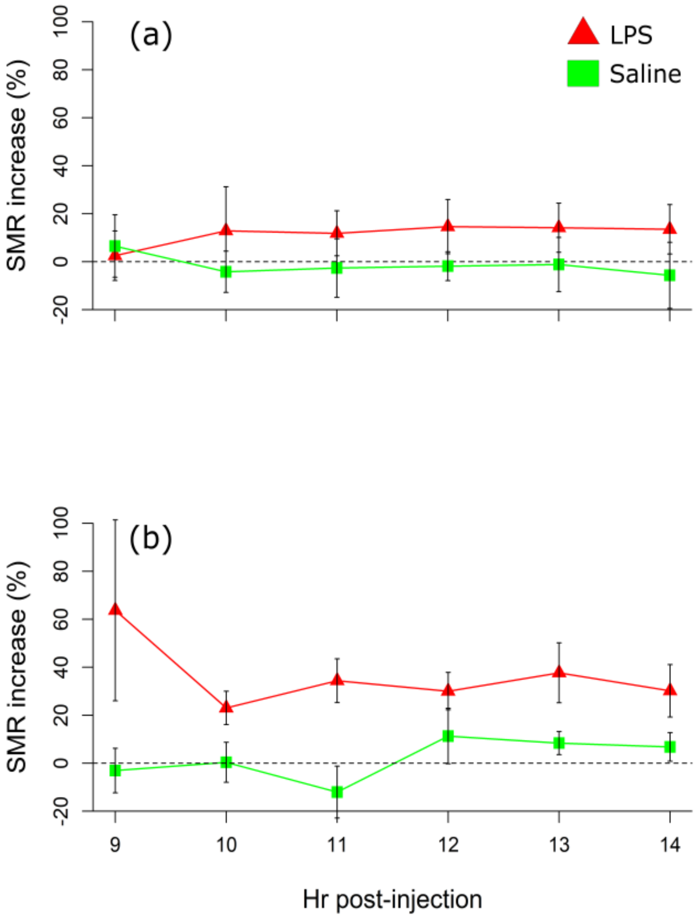

Fig. 2.1 Standard metabolic rate (SMR) increase (%) of Florida cane toads (*Rhinella marina*) injected into the dorsal lymph sac with either LPS (20 µg g^{-1} body mass (strain 0111:B4)) (triangles) or saline (squares) from (A) a northern edge (New Port Richey) (n = 9,6 (LPS and saline)) and (B) a southern core (Miami) population (n = 11,5 (LPS and saline)) compared to baseline metabolic rates from 9-14h post-injection. All metabolic data were collected from toads responding to the treatments at 32°C. Dashed lines indicate no change in SMR compared to baseline trials. The graphs show mean values for each time point and error bars represent ±1 SE

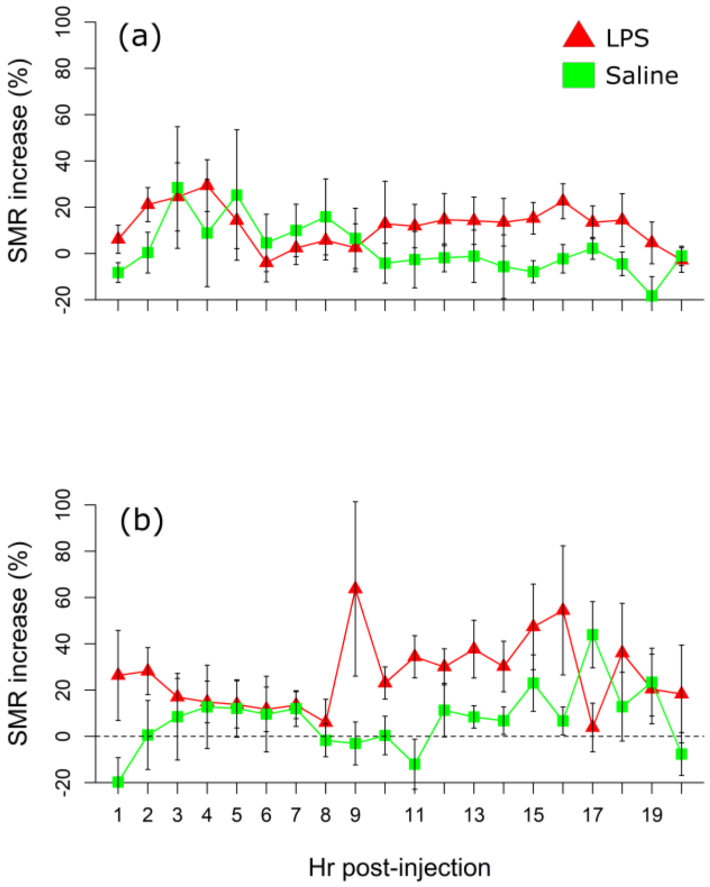

Fig. 2.2 Standard metabolic rate (SMR) increase (%) of Florida cane toads (*Rhinella marina*) injected into the dorsal lymph sac with either LPS (20 µg g^{-1} body mass (strain 0111:B4)) (triangles) or saline (squares) from (A) a northern edge (New Port Richey) (n = 9,6 (LPS and saline)) and (B) a southern core (Miami) population (n = 11,5 (LPS and saline)) compared to baseline metabolic rates for 20 hr post-injection. All metabolic data were collected from toads responding to the treatments at 32°C. Dashed lines indicate no change in SMR compared to baseline trials. The graphs show mean values for each time point and error bars represent ±1 SE

N:L ratios

NL ratios were not affected by sex (n = 21, 36 female and male toads, respectively) (t_{47} = -1.23, P = 1), or CORT (n = 57) (t_{47} = 0.483, P = 0.89). Among the treatments, at 2h there were no differences between LPS groups (n = 12, 10 (edge and core, respectively) (t_{47} = 1.14, P = 1), and NL ratios of LPS-injected cane toads were not different compared to saline-injected toads within either population (n = 12,5 (LPS and saline for the edge population) (t_{47} = 1.75, P = 1), and n = 10,4 (LPS and saline for the core population) (t_{47} = -2.41, P = 0.54), respectively). At 20h core population cane toads responding to LPS (n = 9) had NL ratios that were 0.77 ($\pm$ 0.22) higher compared to saline-injected core individuals (n = 5) (t_{47} = 3.512, P = 0.005), while LPS-injected cane toads from the edge population (n = 7,5 (LPS and saline, respectively) did not (t_{47} = 1.90, P = 1); however, there was no difference in NL ratios between populations injected with LPS (t_{47} = 2.15, P = 0.07) (Fig. 2.3).

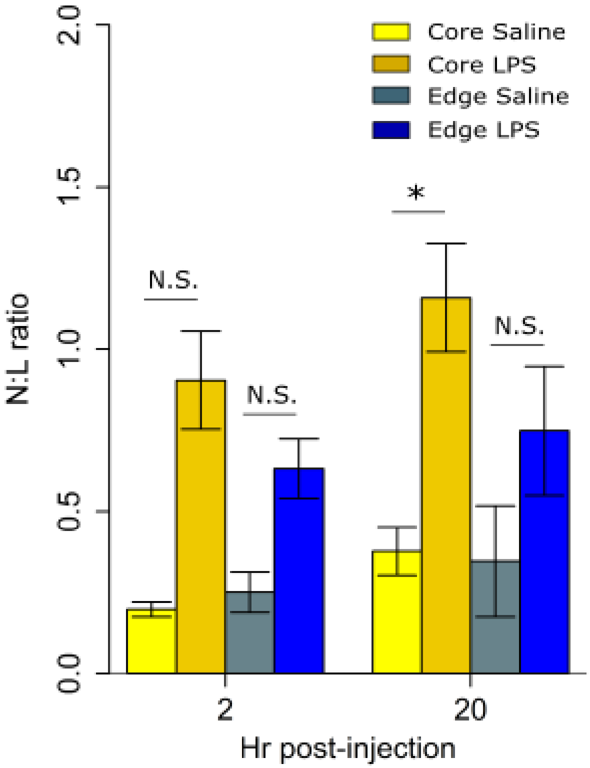

Fig. 2.3 Neutrophil : lymphocyte (N:L) ratios of cane toads (*Rhinella marina*) at 2h and 20h following injections with either amphibian saline or LPS (20 µg g^{-1} body mass (strain 011:B4)) from a southern core (Miami) and northern edge (New Port Richey) population in Florida (n = 4,10 and 5,12 (saline and LPS, core and edge, respectively, at 2h) and n = 5,9 and 5,7 (saline and LPS, core and edge, respectively at 20h)) (* significant effects of $P < 0.05$). The graphs show mean values and error bars represent ± 1 SE, N.S. not significant for treatment effect

BKA

At 2h BKA was not affected by sex (n = 14, 17 (female and male toads, respectively)) (t_{25} = 2.30, P = 0.21), or CORT (n = 31) (t_{25} = 0.26, P = 1). There were no differences in BKA at 2h between LPS-injected toads from edge or core populations (t_{25} = 0.60, P = 1), or between LPS and saline-injected toads within either population (n = 12,6 (LPS and saline for the edge population) (t_{25} = 0.88, P = 1), and n = 9,4 (LPS and saline for the core population) (t_{25} = 1.03, P = 1), respectively). At 20h, BKA was also not affected by sex (n = 6,20 (female and male toads, respectively) (t_{20} = 1.06, P = 1), or CORT (n = 26) (t_{20} = 1.18, P = 1). BKA for edge population cane toads injected with saline (n = 5) was 27.8 (± 8.1) % lower than LPS-injected toads from the edge (n = 8) (t_{20} = -3.45, P = 0.02), although there was no difference in core population BKA between LPS (n = 8) and saline (n = 4) treatment groups (t_{20} = 1.98, P = 0.43). There was no difference in BKA between LPS-injected cane toads between the populations (t_{20} = -1.96, P = 0.45) at 20h (Fig. 2.4).

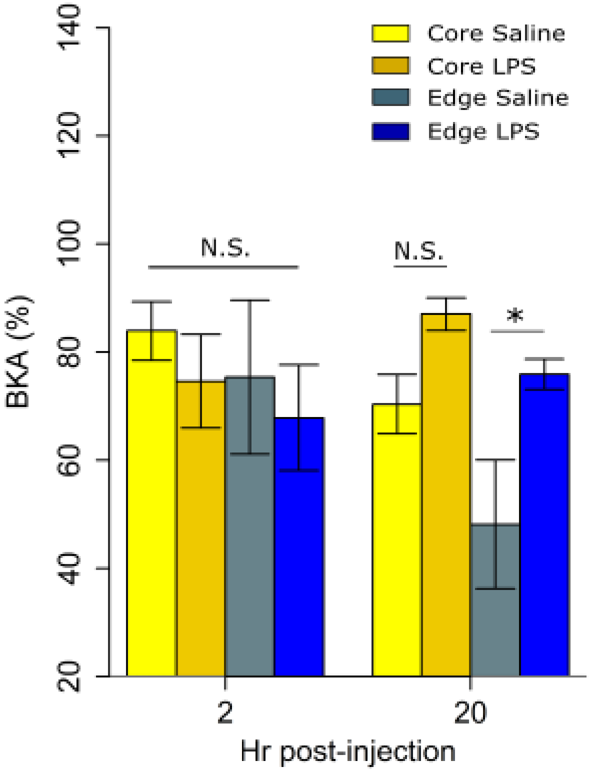

Fig. 2.4 Plasma bacteria killing ability (BKA) of cane toads (*Rhinella marina*) injected with either amphibian saline or LPS (20 µg g^{-1} body mass (strain 011:B4)) at 2h and 20h following injections from a southern core (Miami) and northern edge (New Port Richey) population in Florida (n = 4,9 and 6,12 (saline and LPS, core and edge, respectively) at 2h) and n = 5,8 and 5,8 (saline and LPS, core and edge, respectively) at 20h) (* significant effects of $P < 0.05$). The graphs show mean values and error bars represent ± 1 SE, N.S. not significant for treatment effect

Circulating CORT

At 2h CORT levels were not affected by sex (n = 15, 20 (female and male, respectively)) (t_{30} = -0.58, *P* = 1). There were no differences in CORT between LPS-injected cane toads from either population (n = 12, 11 (edge and core populations, respectively) (t_{30} = 1.42, *P* = 1), and CORT levels of LPS-injected cane toads were not different compared to saline-injected individuals within either population (n = 12,6 (LPS and saline-injected cane toads for the edge population, respectively) (t_{30} = 1.09, *P* = 1), and n = 11,6 (LPS and saline-injected cane toads for the core population, respectively) (t_{30} = 1.16, *P* = 1). At 20h CORT levels were also not affected by sex (n = 10, 21 (female and male toads, respectively)) (t_{26} = 0.003, *P* = 1). Similar to 2h, there were no differences in CORT between LPS-injected cane toads from either population (n = 9, 10 (edge and core populations, respectively)) (t_{26} = 1.06, *P* = 1), and CORT levels were not different within each population between LPS and saline treatment groups (n = 9,6 (LPS and saline-injected cane toads for the edge population, respectively)) (t_{26} = 0.994, *P* = 1), and n = 10,6 (LPS and saline-injected cane toads for the core population, respectively)) (t_{26} = 1.10, *P* = 1) (Fig. 2.5).

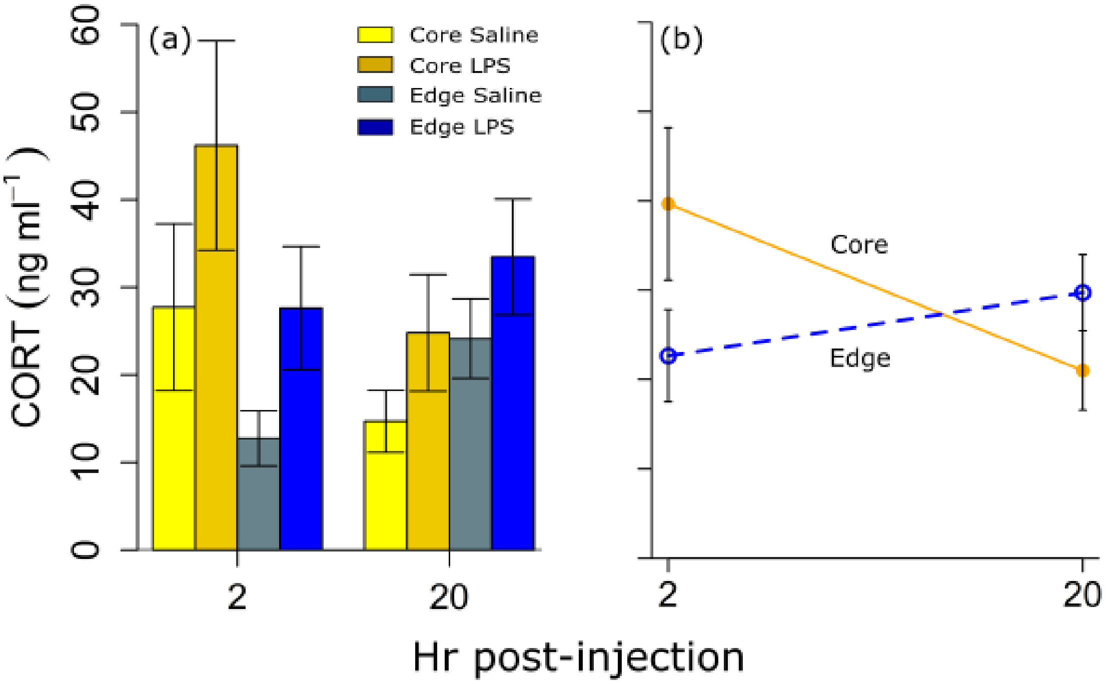

Fig. 2.5 Circulating corticosterone levels (CORT) of cane toads (*Rhinella marina*) (A) injected with either amphibian saline or LPS (20 µg g^{-1} body mass (strain 011:B4)) at 2h and 20h following injections from a northern edge (New Port Richey) and southern core (Miami) population in Florida (n = 6,11 and 6,12 (saline and LPS, core and edge, respectively) at 2h) and n = 6,10 and 6,9 (saline and LPS, core and edge, respectively at 20h)). There was an interaction between time and location (*P* = 0.03) (B), such that CORT decreased in core-population cane toads (n = 17, 16 (2h and 20h, respectively)) (closed circles) with time compared to edge-population toads (n = 18,15 (2h and 20h, respectively)) (open circles). The graphs show mean values and error bars represent ± 1 SE. N.S. not significant for treatment effect

Discussion

When compared to individuals from the saline-treatment group, neither core or edge population receiving LPS had elevated metabolic rates, and the rates between populations responding to the LPS injections were also not different. This is likely due to high individual variation (Careau et al. 2008); however, when comparing both treatment groups from the two populations to their baselines only core individuals injected with LPS had elevated metabolic rates, the most pronounced period of which was from 9-14h. The significant elevation in metabolic rates observed only when comparing LPS-injected individuals to their baseline levels may reflect a benefit of repeated-measures studies in which immune responses of individuals are compared to their baseline values. The response change then being compared among treatments, populations, etc. (discussed by Selechnik et al. 2017), then takes into account any differential responses and variation within individuals and populations (Careau et al. 2008). The period of metabolic increase in LPS-injected toads from the core population is similar to the results of Sherman and Stephens (1998), in which cane toads had elevated rates 9h following injections. The increase in metabolic rates of the core population LPS-injected individuals is similar to two other studies involving invasive amphibians, one from the study by Llewellyn et al. (2012), in which captive-raised juvenile Australian cane toads born from parents along an invasion front gradient had decreasing metabolic rates with increasing mid-parent distance from the core population following stimulus with LPS (although those individuals were 53.5 ± 1.9 g, approximately half the size of toads used in this study). The other study showed that Cuban tree frogs from southern core populations in southern Florida had elevated metabolic rates following 24h post-injection with LPS compared to individuals from the expanding northern range in Florida (Goetz et al. 2017). Cane toads from the core population having significantly higher metabolic changes compared to those from the edge population indicates differential energy allocation to an innate immune challenges occurs between these populations, which may be explained by the inhibitory effect of elevated GCs on pro-inflammatory gene expression (Padgett and Glaser 2003) for toads from the edge, although the study by Gardner et al. (2018) showed that toads from the same edge population do have significant gene expression of pro-inflammatory cytokines at 2h following LPS injections.

Increases in circulating neutrophils following infections or immune responses in amphibians have been well documented (Brown et al. 2011; Peterson et al. 2013; Gervasi et al. 2014; Greenspan et al. 2017), although only LPS-injected toads from the core population showed elevated ratios after 20h post-injection with LPS (compared to saline-injected toads), and there was no difference in NL ratios in LPS-injected toads between the two populations. These results are similar to wild caught Australian cane toads injected with the same dose of LPS used in our study (Selechnik et al. 2017), although in the Australian toads, blood samples were collected 14 days post-injection. A potential explanation for the lack of significance between these groups, as well as saline-injected individuals from the edge population not having lower NL ratios compared to the LPS-injected cane toads from the edge population could be due to the increasing CORT levels in this population with time. Although CORT levels did not have a significant effect on NL ratios of cane toads at the time points measured (2 and 20h), it has been shown that elevated GC levels affect NL ratios (Davis et al. 2008) by increasing extravasation of circulating lymphocytes into tissues (Dhabhar 2002), while also increasing influxes of neutrophils from bone marrow into circulation (Bishop 1968). This has been documented in amphibians (Bennett and Alspaugh 1964) which could be contributing to the variability in ratios in this study.

Compared to metabolic rates and NL ratios, LPS-injected individuals did not have elevated BKA compared to saline-injected toads from the core population or LPS-injected individuals from the edge population. Instead, BKA decreased with time in the saline treatment group, and at 20h post-injection saline-injected toads had significantly lower BKA compared to LPS-injected individuals within the edge population. CORT increased over time in these individuals, and elevated GC levels have an inhibitory effect on complement activity (Packard and Weiler 1983) through mechanisms involving the inhibition of convertase (Gewurz et al. 1965; Jennings and Taylor 1964; Weiler and Packard 1982). This inhibitory effect of GCs on BKA has also been shown in Australian cane toads (Graham et al. 2012). Selechnik et al. (2017) indicated that the timing of immune response may be the difference between populations of invasive species at the leading edge of an invasion compared to core populations. Although not significant for the two time points measured in this study, regressions of CORT against BKA or NL ratios in individuals from core and edge populations over multiple time points may allow for further testing of this hypothesis.

The cane toads measured in this study from the southern core population had CORT values that were initially greater (although not significant) than those from the northern edge population, similar to the study by Brown et al. (2015) who found that cane toads from more recently established Australia populations had decreased CORT responses compared to longer-established populations when subjected to the same stressor. Compared with field levels from a different subset of individuals from both populations obtained one month earlier in the season (3.87 (± 0.68) ng ml^{-1} and 1.29 (± 0.15) ng ml^{-1} for core and edge, respectively (unpublished data)), edge cane toads had a higher initial CORT response for both saline and LPS treatments; however, as toads were not sampled prior to being placed in the metabolic chambers, it is unclear if this change from field levels is solely a response to the treatments, or a combination of the time of when these individuals were collected from the field, differences among subsets of these toads from the same populations, an effect of captivity, or combinations of these variables. Regardless of the differences between lab and field hormone levels, CORT in both treatment groups from the core population decreased with time, while CORT in cane toads from the edge population increased over time, indicating individuals from these populations responds differently to novel stimuli. This difference may be due to behavioral or "personality" differences (Careau et al. 2008) between the two populations, which have been predicted to be due to differences in reactivity of the neuroendocrine systems of individuals (Koolhaas et al. 1999). The difference in stress response may be beneficial for edge compared to core populations. Increased CORT levels may attenuate or alter the timing of immune responses (Selechnik et al. 2017), potentially leading to higher susceptibility to infection (Rollins-Smith 2001); however, this may come at less of a cost in expanding populations, as a result of having lowered parasitic and pathogen loads in novel habitats near the leading-edge (Phillips et al. 2010). The costs associated with fighting an infection taking longer to accumulate in leading-edge cane toads could be more favorable to survival by reducing energetic costs that could require an increase in prey items and time spent foraging, which could therefore increase vulnerability to predation, where the native predators may have not yet learned to avoid them (Shine 2010). This response could be more advantageous on the leading-edge compared to longer-established core populations of cane toads that have been present long enough that native predators have learned to avoid them, and where there is a greater prevalence of parasites and pathogens that larger immune responses are required for dealing with.

These results support the hypotheses that immune responses of invasive species are affected from dispersal into novel habitats; however, more attention to mechanisms behind differential stress profiles in these organisms and their effect on immune responses is needed. The results of this study and the multiple studies on Australian cane toad populations, indicate that differential stress responses associated with populations experiencing higher levels of unpredictability or harsher conditions may be a main contributor to their varied immune responses (Rollins-Smith 2017). Previous work with amphibians has shown that increased stress levels early in development may lead to decreased responsiveness to GCs in GC-reactive brain regions and in their anterior pituitary glands (Hu et al. 2008), altering sensitivity to stress later in life (Yao et al. 2008). Future work assessing epigenetic modifications of GC receptors and cytokine expression levels in these populations following immune stimuli, as well as ecological studies showing the types of increased pressures in leading-edge populations of cane toads, whether increased predation risk, higher selective pressures during early life stages, or tradeoffs with habitat quality and further dispersal, would be beneficial for illustrating how stress, and as a result immune responses, have been affected by northward dispersal among the populations in Florida.

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Chapter 3

Differences in morphology and in composition and release of parotoid gland secretion in introduced cane toads (*Rhinella marina*) from established populations in Florida, USA

(Formatted for Ecology and Evolution)

The final publication is available at:

https://onlinelibrary.wiley.com/doi/full/10.1002/ece3.7118

Abstract

Cane toads are highly toxic bufonids invasive in several locations throughout the world. Although physiological changes and effects on native predators for Australian populations have been well documented, Florida populations have received little attention. Cane toads were collected from populations spanning the invaded range in Florida to assess relative toxicity, through measuring morphological changes to parotoid glands, likelihood of secretion, and the marinobufagenin (MBG) content of secretion. We found that residual body indices increased in individuals from higher latitude populations, and relative parotoid gland size increased with increasing toad size. There was no effect of latitude on the allometric relationship between gland size and toad size. We observed an increase in likelihood of secretion by cane toads in the field with increasing latitude. Individuals from southern and northern populations did not vary significantly in the quantity of MBG contained in their secretion. Laboratory-acclimated cane toads receiving injections of epinephrine were more likely to secrete poison with increasing dose, although there was no difference in likelihood of secretion between southern and northern populations. This suggests that differences between populations in the quantities

of epinephrine released in the field, due to altered hypothalamic sensitivity upon disturbance, may be responsible for the latitudinal effects on poison secretion. Our results suggest that altered pressures from northward establishment in Florida have affected sympathetic sensitivity and defensive mechanisms of cane toads, potentially affecting risk to native predators.

Introduction

Cane toads (*Rhinella marina*) are large and highly toxic members of the family Bufonidae and are native to South America (Zug and Zug 1979; Acevedo et al. 2016). Due to their voracious feeding responses, cane toads were introduced into Australia in the early to mid-1900's as a means of controlling sugar cane pests (Lever 2001; Phillips et al. 2006) and were released in Florida (Miami) in the United States prior to 1955 (Krakauer, 1968). They have dispersed in these locations with invasion rates of up to 55 km yr^{-1} in Australia (Phillips et al. 2007). Additionally, *R. marina* reproduce in large numbers, and females may lay up to 30,000 eggs per clutch in invaded areas (Hagman and Shine 2006). However, the extent of cane toad dispersal and reproductive capacity in the United States, where they have established in higher latitude temperate areas (Mittan & Zamudio, 2019) is not well known.

Much of the devastation to native Australia fauna and predators caused by invasive cane toads has been due to their highly toxic secretions, common to bufonids and many other amphibians (Garg et al., 2007), that significantly impact the survivorship of native predators from the areas they invade (Shine, 2010b). Toad poison is synthesized and stored in the large parotoid glands on the shoulders (Mailho-Fontana et al. 2014) and in other cutaneous macroglands in the skin (Pedro Luiz Mailho-Fontana et al., 2018). The secretion contains a cocktail of toxins (Hayes et al., 2009b), the main components of which belong to a family of compounds known as bufadienolides (BDs) (Chen and Kovaříková 1967; Lever 2001). These steroidal compounds, derived from cholesterol (Porto & Gros, 1971), are more broadly classified as cardiotonic steroids (Steyn and van

Heerden 1998; Sousa et al. 2017). These compounds exert their effects by binding to the enzyme Na^+/K^+-ATPase to induce sustained contraction. Marinobufagenin (MBG), is a potent cardiotonic compound found in the secretions of *R. marina* (Sciani et al., 2013), and has been shown to induce cell death in cardiac myocytes (Liu et al., 2012).

Altered selective pressures may affect various physiological aspects in amphibians, including the potency of poisonous secretions. Members of the toad species *Bufo bufo* occupying regions of elevated anthropogenic disturbance differ in toxicity compared to those in less disturbed agricultural areas (Bókony et al., 2019), and individuals of *Bufo boreas* exposed to increased predation cues prior to metamorphosis had higher concentrations of more toxic compounds in their secretions (Benard & Fordyce, 2003). Altered morphological and physiological changes have been documented in *R. marina* at the invasion front when compared to longer established populations (Friesen & Shine, 2019; Gardner et al., 2020). Parotoid gland size has been shown to increase with body size (snout-vent length (SVL)) in *R. marina* (Phillips and Shine 2005; Phillips et al. 2007). In Australia this allometric increase is greater in toads at the invasion front than in long-established populations (Ben L. Phillips & Shine, 2005). This may indicate that cane toads produce more toxic secretions when invading new habitats, possibly to deter predators early in the invasion process, as many predators rapidly learn to avoid preying on toads (Greenlees et al. 2010).

Prior to the introduction of *R. marina*, Australia had no toad species (Beckmann & Shine, 2009; Hayes et al., 2009b). In contrast, potential predators that may encounter cane toads invading Florida have evolved in sympatry with other toad species, such as the southern toad, *Anaxyrus terrestris*, which also possess BDs in their toxic secretions (Mohammadi et al., 2016). Invertebrate predators in Florida are capable of consuming cane toad eggs with low mortality risk (Punzo & Lindstrom, 2001) relative to larval anuran predators and fish. Other native predators, including several snake species, opossums, and birds (Meshaka 2011) have been observed consuming adult toads with little or no ill-effect.

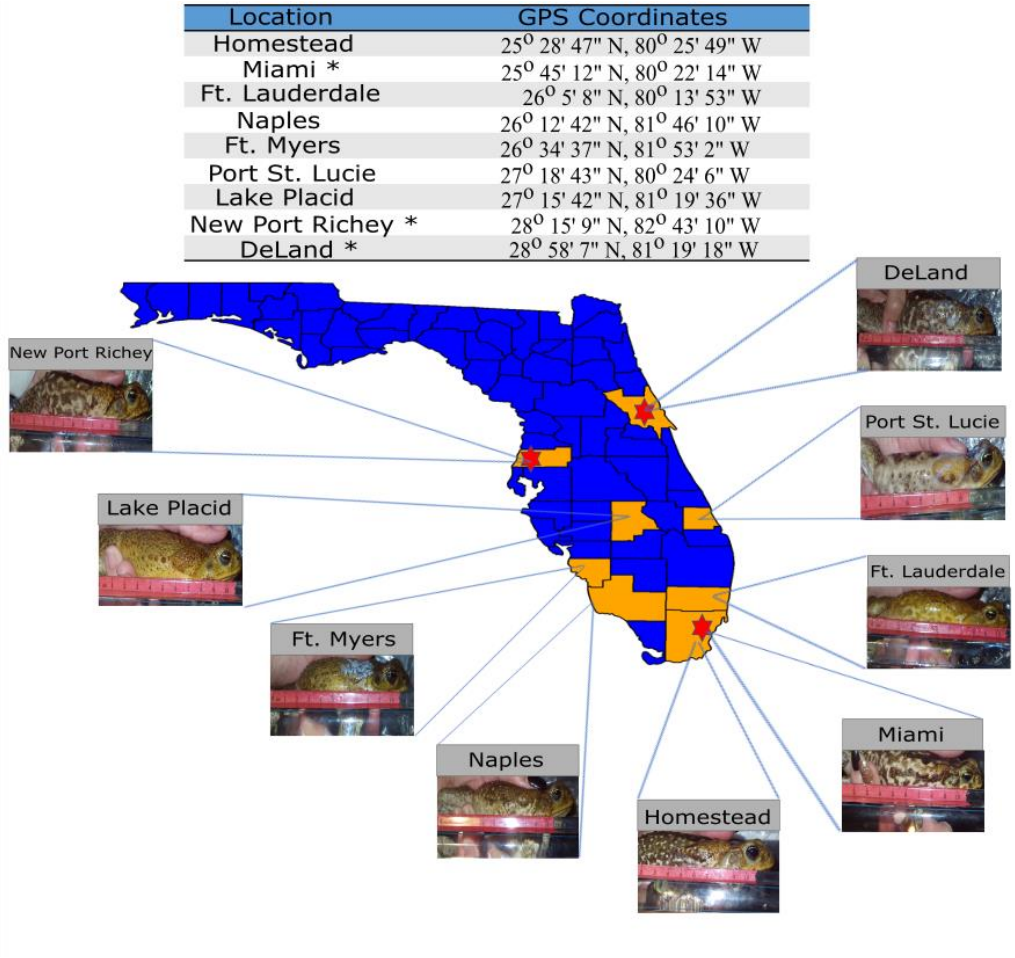
Individuals from edge populations of *R. marina* in Australia have shown altered behavior to novel stressors, such as decreased escape behavior and an increased likelihood of secreting poison during simulated predation events (Hudson et al., 2017). Although these behaviors have yet to be observed for toads in Florida, toads near the northern edge populations have shown elevated baseline corticosterone concentrations and attenuated responses to a restraint challenge when compared to individuals from southern core populations (Assis et al. 2020). This pattern of attenuated response to novel stressors has also been documented under laboratory conditions for Florida cane toads (Gardner et al., 2020).

Although the risk of *R. marina* poisoning among native predators in Australia has been well-documented (Crossland et al. 2008; Greenlees et al. 2006; Letnic et al. 2008; Phillips and Shine 2006), that risk has not been reported quantitatively for the Florida cane toad invasion. Here we assess morphological, behavioral, and physiological characteristics reflecting toxicity risk of *R. marina* from well-established sites in southern Florida and from sites stretching northward to the invasion front. We measured the size of the parotoid glands, the likelihood of secretion, and the concentration of MBG in samples of secretion obtained in the field. We then collected toads to assess the epinephrine-induced secretion response under controlled laboratory conditions. We chose to use MBG concentration as an index of overall toxicity of cane toads from field samples obtained from southern and northern populations due to the potency and abundance of this compound in the integumentary secretion of *R. marina*. We also document morphological and physiological differences between the source populations in southern Florida and established populations occurring closer to the invasion front in northern Florida.

Methods

Field Sampling, 2018

Approximately 20 toads from nine populations (established from 1955 to 1991 (U.S. Geological Survey 2020)) across a south – north latitudinal gradient representing the current invasive range in Florida were collected from 10 – 19 May 2018. Toads were collected from approximately 2000-2400 h and placed into plastic bags immediately upon capture (Fig. 3.1) (for exact numbers collected from each population, see Table 3.1). One hour following capture, toads were removed from the bags and their mass, sex, and snout-vent-length (SVL) were recorded. Toads were then photographed next to a ruler for scale, and the images were used to measure gland size (right parotoid gland), as well as to indicate whether a toad was secreting poison following the capture and handling period. Measurements of total length, width, and area of glands were performed using ImageJ software.



Location	GPS Coordinates
Homestead	25° 28' 47" N, 80° 25' 49" W
Miami *	25° 45' 12" N, 80° 22' 14" W
Ft. Lauderdale	26° 5' 8" N, 80° 13' 53" W
Naples	26° 12' 42" N, 81° 46' 10" W
Ft. Myers	26° 34' 37" N, 81° 53' 2" W
Port St. Lucie	27° 18' 43" N, 80° 24' 6" W
Lake Placid	27° 15' 42" N, 81° 19' 36" W
New Port Richey *	28° 15' 9" N, 82° 43' 10" W
DeLand *	28° 58' 7" N, 81° 19' 18" W

Fig 3.1 Cane toad populations sampled in 2018 for morphological measures and likelihood of secretion. Locations are listed above images of collected cane toads, with images depicting representative toads from each sampled location being recorded for gland sizes following mass, sex, and SVL being recorded. Toads were collected along a south to north gradient, from Homestead to DeLand, FL. Sites indicated by a star indicate populations sampled in 2019 for MBG concentration in poison secretions.

Field Sampling, 2019

In May of 2019, toads from a southern source population in Miami (n = 10), as well as two northern "edge" populations, New Port Richey (NPR) and Deland (n = 10 NPR, n = 7 DeLand), were captured. Secretion samples were collected into cryovials by gentle compression of the parotoid glands (R. C. Toledo et al., 1992) and immediately frozen in liquid nitrogen. Sex, SVL, mass, and parotoid gland measurements were recorded for each toad sampled.

Marinobufagenin content of samples

Samples were kept frozen from the time of field collection through their transport to the lab. The lids of vials containing parotoid secretion were punctured with a 16G needle and the contents freeze dried (LABCONCO Freezone 4.5). The dry contents were removed after 24 hours, and the dry mass of each whole sample was recorded. A portion of each sample was transferred to a glass vial and diluted with 0.5ml mg^{-1} of a 90:10 methanol/water UV grade solution. The samples were then sonicated for 30 min, rotated, and sonicated for another 15 min. Following sonication, the samples were centrifuged for 15 min at 3000 rpm to separate undissolved solid material. The supernatant was then collected and stored for analysis in a glass instrument vial (1.5 mL) at $-20^{o}C$.

High performance liquid chromatography (HPLC) was carried out on a Shimadzu LC-2030, equipped with an Econsil C18 column (250 x 4.6 mm, i.d.; particle size: 10 um) from Alltech. The column was maintained at room temperature and run at a pump speed of 1 mL min^{-1}. The mobile phases consisted of A = milli-Q water with 0.1%

trifluoracetic acid and B = acetonitrile + 0.1% TFA. Gradient methodology was employed using the following method: 20% B for 1 min then raised to 42% over 42 min. It was then raised to 95% B over 0.1 and held there for 4.9 min. B was lowered to 20% in 0.1 min and held for 3.9 min to return the column to equilibrium.

A commercial standard of marinobufagenin (MBG; Cayman Chemicals, catalog # 20798) was reconstituted in methanol. The peak was first identified at 40.45 min. For verification, test samples separate from those analyzed were spiked with commercially acquired marinobufagenin, and the additional peak height was used to confirm the identity of the marinobufagenin peak using the elution protocol described.

The MBG peak for each sample occurred at approximately 40.45 min per the elution gradient described above. The area of each peak was divided by the mass of the dry sample and the peak/mass ratio was used in further analysis. Peak tailing was minimal after the optimal gradient elution protocol was established. However, area under the curve was chosen over peak height to accommodate any minor peak tailing or broadening. MBG sample concentrations were determined by the use of a four-parameter standard curve. Using the integrated peak value and a five-point calibration plot, the concentration of MBG (ug/mg) in solution was determined. This concentration was then back-calculated relative to the amount of solution used for sample preparation and adjusted for the dry weight of each sample prior to extraction.

Sympathetic sensitivity

In May 2019, 58 additional toads (n = 28 Miami, and n = 30 NPR) were collected and maintained under laboratory conditions, detailed by Gardner et al. (2020). Following acclimation, the toads were divided into five treatment groups: normal saline solution (NSS) and NSS with 0.125 µm, 0.25 µm, 0.5 µm, or 1 µm epinephrine gram of body mass^{-1}. Each treatment group was comprised of 6 toads collected from Miami and NPR (with the exception of the 0.125 µm treatment group, which had only 4 toads from Miami). Following injections, each toad was placed in a plastic bin (60.96 x 46.99 x 40 cm) and monitored for signs of poison secretion.

Statistical Analyses

To compare the morphological parameters in Florida populations of cane toads with those observed in a previous study by Phillips and Shine (2005), a model using the lm function in R (version 3.6.3), using latitude coordinates (decimal degrees) as a covariate and sex as a factor, was used to assess differences in $\log_{10}$-transformed SVL measurements of toads. Log_{10}-transformed body masses of toads collected from the 9 populations sampled during 2018 were then regressed against SVL measurements (also $\log_{10}$-transformed) to obtain residual body condition index measures (Denoël et al., 2002). This measure follows a normal distribution that is independent of SVL (Plăiaşu et al., 2010) and has commonly been used to assess fitness and performance of individuals from different populations (Scheele et al., 2014; Unglaub et al., 2018). A model using sex as a factor and latitude and $\log_{10}$-transformed SVL (toad size) as covariates was used to assess differences in Body Index (BI) for the 2018 dataset.

Parotoid gland sizes were evaluated by performing a Principal Component Analysis (PCA) (using the princomp function in R) on $\log_{10}$-transformed width, length, and area of glands obtained from images taken in the field. The first principal component, termed “gland size” and characterized by parotoid gland length, area, and width, accounted for 60.19% of the variation in gland measurements, and was used in a model (using the lm function in R) with latitude, BI, and toad size as covariates and sex as a factor, to assess differences in gland sizes among the 9 populations sampled in 2018. An interaction term between toad size and latitude was originally included in this model; however, as this term was not significant ($P > 0.05$), it was removed. An additional model (using the glm function from the nlme package in R) using sex as a factor and BI, gland size, toad size, and latitude as covariates was used to assess likelihood of the toads secreting poison in the field during 2018. BI was obtained for toads collected in 2019 from Miami, New Port Richey, and DeLand (as well as gland measurements), and concentration of MBG in collected secretions was assessed using BI, gland sizes, and toad size as covariates and location as a factor. The likelihood of the toads secreting poison following 3 weeks of laboratory acclimation was assessed using epinephrine dose as a covariate and location as a factor for toads collected in 2019. Resulting *P* values from all analyses were adjusted using Bonferroni correction in R.

Results

Morphology and likelihood of secretion (2018)

There was no effect of latitude (-0.008 (± 0.004), t_{176} = -2.06, $P = 0.08$) or sex (0.002 (± 0.008), t_{176} = 0.24, $P = 1.0$ for 110 males compared to 69 females) on sizes of cane toads collected during 2018 (values represent mean (±) standard error). BI of male toads measured in 2018 did not differ from females (-0.006 (± 0.010), t_{175} = -0.638, $P =$ 1.0), and BI was also not affected by toad size (0.055 (± 0.09), t_{175} = 0.59, $P = 1.0$). BI did however increase in collected toads by 0.020 (± 0.005) (t_{175} = 3.87, $P < 0.01$) with each degree of increasing latitude among the cane toad populations. Parotoid gland size was not affected by BI (0.57 (± 0.24), t_{174} = 2.37, $P = 0.08$), or latitude (-0.010 (± 0.017), t_{174} = 0.58, $P = 1.0$). BI was also not different for male compared to female toads (0.011 (± 0.034), t_{174} = 0.32, $P = 1.0$), although gland size increased significantly (1.92 (± 0.30), t_{174} = 6.50, $P < 0.01$) with increasing toad size (for the full list of sample sizes, means, standard errors, data ranges, and medians for morphological data collected in 2018 see Table 3.1). There was no effect of BI (0.200 (95% C.L. = 0.0005 – 72.84), z_{173} = 0.55, P = 1.0), gland size (2.48 (95% C.L. = 0.54 – 11.28), z_{173} = 1.20, $P = 1.0$), or toad size (2.09 (95% C.L. = 0.0007 – 6022.76), z_{173} = 0.19, $P = 1.0$) on the likelihood of a toad to secrete poison upon capture and handling. Male toads were also no more likely to secrete poison compared to female cane toads (3.03 (95% C.L. = 1.11 – 8.24), z_{173} = 2.22, $P =$ 0.13); however, cane toads were 1.94 (95% C.L. = 1.29 – 2.91; z_{173} = 3.24, $P < 0.01$) times more likely to secrete poison with each degree of increasing latitude (Fig. 3.2).

Table 3.1. Cane toad morphological data (2018)

Variable	Location	Number of individuals	Range	Median	Mean	St. dev	St. err
Mass (g)	Homestead	21	70.00 – 292.00	137.00	141.43	57.18	12.48
	Miami	20	70.00 – 271.00	118.50	131.40	50.55	11.30
	Ft. Lauderdale	20	44.00 – 158.00	93.50	98.70	31.5	7.04
	Naples	20	53.00 – 211.00	112.00	109.60	39.06	8.73
	Ft. Myers	25	76.00 – 165.00	117.00	119.72	28.64	5.73
	Port St. Lucie	20	38.00 – 235.00	102.50	110.90	44.6	9.97
	Lake Placid	20	53.00 – 150.00	90.50	96.25	30.48	6.82
	New Port Richey	20	63.00 – 308.00	100.25	122.25	60.29	13.48
	DeLand	13	44.00 – 386.00	123.00	153.00	98.32	27.27
SVL (mm)	Homestead	21	91.90 – 148.00	111.60	112.53	13.65	2.98
	Miami	20	90.00 - 189.70	108.40	113.00	21.06	4.71
	Ft. Lauderdale	20	79.20 - 120.40	98.90	99.68	9.71	2.17
	Naples	20	87.50 - 132.10	107.20	106.50	11.94	2.67
	Ft. Myers	25	92.50 - 122.70	108.50	108.21	8.75	1.75
	Port St. Lucie	20	70.30 - 121.60	103.10	101.99	11.66	2.61
	Lake Placid	20	81.50 - 115.90	98.90	97.79	10.85	2.43
	New Port Richey	20	83.50 - 145.20	97.80	102.63	16.42	3.67
	DeLand	13	75.10 - 148.40	105.90	109.34	18.26	5.06
Residual Body Index	Homestead	21	-0.12 - 0.09	0.02	0.00	0.06	0.01
	Miami	20	-0.54 - 0.10	< 0.01	-0.02	0.14	0.03
	Ft. Lauderdale	20	-0.12 - 0.07	< 0.01	-0.01	0.05	0.01
	Naples	20	-0.17 - 0.07	< 0.01	-0.04	0.06	0.01

	Ft. Myers	25	-0.11 - 0.05	< 0.01	-0.01	0.04	0.01
	Port St. Lucie	20	-0.09 - 0.29	< 0.01	0.01	0.08	0.02
	Lake Placid	20	-0.06 - 0.08	0.01	0.01	0.04	0.01
	New Port Richey	20	-0.10 - 0.13	0.05	0.04	0.05	0.01
	DeLand	13	-0.08 - 0.16	0.03	0.04	0.06	0.02
Gland width (cm)	Homestead	21	1.10 - 1.94	1.5	1.54	0.24	0.05
	Miami	20	1.06 - 1.78	1.38	1.37	0.18	0.04
	Ft. Lauderdale	20	0.81 - 1.64	1.31	1.28	0.19	0.04
	Naples	20	0.97 - 2.01	1.39	1.42	0.25	0.06
	Ft. Myers	25	1.13 - 3.30	1.41	1.51	0.42	0.08
	Port St. Lucie	20	1.04 - 1.86	1.5	1.49	0.23	0.05
	Lake Placid	20	0.94 - 1.71	1.36	1.37	0.23	0.05
	New Port Richey	20	1.06 - 2.00	1.28	1.39	0.28	0.06
	DeLand	13	0.84 - 2.05	1.25	1.31	0.33	0.09
Gland length (cm)	Homestead	21	2.10 - 4.40	2.89	2.91	0.53	0.12
	Miami	20	1.92 - 3.25	2.64	2.70	0.33	0.07
	Ft. Lauderdale	20	1.78 - 2.72	2.38	2.38	0.24	0.05
	Naples	20	2.16 - 3.09	2.48	2.56	0.27	0.06
	Ft. Myers	25	2.05 - 3.51	2.46	2.57	0.37	0.07
	Port St. Lucie	20	1.77 - 3.40	2.74	2.70	0.43	0.10
	Lake Placid	20	1.34 - 3.19	2.47	2.46	0.40	0.09
	New Port Richey	20	2.08 - 3.83	2.47	2.62	0.44	0.10
	DeLand	13	1.98 - 3.45	2.81	2.76	0.41	0.11
Gland area	Homestead	21	1.61 - 5.14	2.67	2.98	0.96	0.21

	Miami	20	1.57 - 3.68	2.23	2.39	0.60	0.13
	Ft. Lauderdale	20	1.08 - 2.82	1.93	1.94	0.47	0.11
	Naples	20	1.43 - 4.69	2.29	2.40	0.71	0.16
	Ft. Myers	25	0.93 - 3.74	2.24	2.27	0.62	0.12
	Port St. Lucie	20	1.12 - 4.31	2.64	2.67	0.82	0.18
	Lake Placid	20	1.34 - 3.47	2.22	2.23	0.60	0.13
	New Port Richey	20	1.29 - 5.21	1.83	2.30	1.01	0.23
	DeLand	13	1.21 - 4.60	2.45	2.52	0.96	0.27
Gland size	Homestead	21	-0.2 - 0.41	0.06	0.09	0.16	0.03
	Miami	20	-0.23 - 0.22	-0.03	-0.01	0.12	0.03
	Ft. Lauderdale	20	-0.42 - 0.08	-0.11	-0.12	0.12	0.03
	Naples	20	-0.24 - 0.32	-0.03	-0.02	0.13	0.03
	Ft. Myers	25	-0.24 - 2.34	-0.04	0.11	0.52	0.10
	Port St. Lucie	20	-0.39 - 0.28	0.05	0.03	0.17	0.04
	Lake Placid	20	-0.30 - 0.18	-0.03	-0.06	0.14	0.03
	New Port Richey	20	-0.30 - 0.40	-0.12	-0.05	0.19	0.04
	DeLand	13	-0.20 - 0.41	0.02	-0.01	0.19	0.05

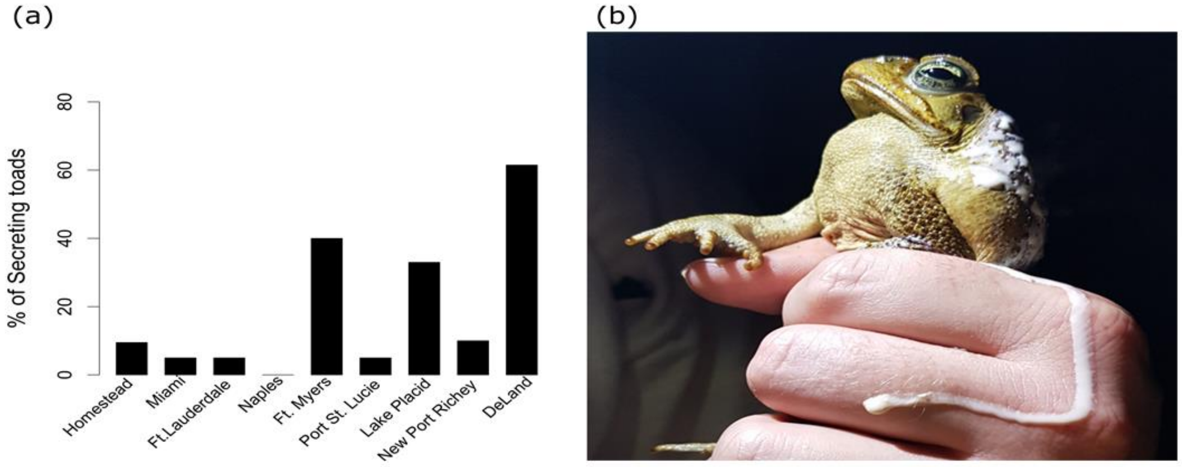


Fig 3.2 Poison secretion data collected from 9 cane toad populations spanning a south to north gradient in FL (*n* = 21, 25, and 13 for Homestead, Ft. Myers, and DeLand, and *n* = 20 for all other populations). Panel (a) represents percentages of toads secreting poison following 1 hr of capture and handling. The likelihood of a toad secreting poison following collection in the field increased significantly (z173 = 3.24, *p* < .01) with increasing latitude, from the most southern population (Homestead) to the most northern (DeLand, FL). Panel (b) depicts a toad secreting poison immediately following capture from a northern FL population (DeLand).

MBG concentration and sympathetic sensitivity (2019)

MBG concentrations assessed using HPLC were not affected by BI (247.34 (± 185.45), $t_{21} = 1.33$, P = 1.0), toad size (469.32 (± 317.21), $t_{21} = 1.48$, P = 0.92), or gland size (-157.10 (± 100.70), $t_{21} = -1.56$, $P = 0.80$). MBG concentrations for Miami cane toads were not different compared to those from NPR (47.38 (± 20.87), $t_{21} = -2.27$, $P = 0.21$), or DeLand (-7.43 (± 29.57), $t_{21} = 0.25$, $P = 1.0$). MBG concentrations were also not different for NPR compared to DeLand cane toads (54.81 (± 30.91), $t_{21} = 1.77$, $P = 0.54$) (for the full list of sample sizes, means, standard errors, data ranges, and medians for morphological and MBG concentration data collected in 2019 see Table 3.2). Although laboratory-acclimated toads (n = 28 Miami, 30 NPR) were 9.54 (95% C.L. = 1.80 – 50.58; $z_{55} = 2.71$, $P = 0.01$) times as likely to secrete poison with increasing epinephrine dose (Fig. 3.3), the likelihood of cane toads secreting poison was not affected by locality (0.76 (95% C.L. = 0.23 – 2.55), $z_{55} = -0.45$, $P = 1.0$).

Table 3.2. Cane toad morphological and MBG concentration data (2019)

Variable	Location	Number of individuals	Range	Median	Mean	St. dev	St.err
Mass (g)	Miami	10	97.00 – 183.00	144.00	144.20	30.96	9.79
	New Port Richey	10	87.00 – 224.00	168.50	168.30	42.14	13.33
	DeLand	7	17.00 – 169.00	86.00	76.43	59.35	22.43
SVL (mm)	Miami	10	96.70 - 126.70	113.15	111.48	9.64	3.05
	New Port Richey	10	96.60 - 137.20	115.15	116.56	12.57	3.97
	DeLand	7	57.20 – 117.20	97.70	87.01	24.93	9.42
Residual Body Index	Miami	10	-0.12 - 0.06	< 0.01	< 0.01	0.05	0.02
	New Port Richey	10	-0.06 - 0.11	0.02	0.01	0.05	0.02
	DeLand	7	-0.06 – 0.01	-0.03	-0.02	0.02	0.01
Gland Width (cm)	Miami	10	1.11 - 2.06	1.63	1.57	0.28	0.09
	New Port Richey	10	1.35 - 2.04	1.79	1.78	0.20	0.06
	DeLand	7	0.88 – 1.77	1.35	1.31	0.43	0.16
Gland length (cm)	Miami	10	2.22 - 3.71	2.89	2.96	0.49	0.15
	New Port Richey	10	2.33 - 3.81	3.27	3.24	0.42	0.13
	DeLand	7	1.28 – 3.31	2.61	2.37	0.85	0.32
Gland area	Miami	10	1.78 - 4.39	2.70	3.00	0.84	0.27
	New Port Richey	10	1.80 - 4.44	3.50	3.28	0.84	0.27
	DeLand	7	0.61 – 3.37	1.92	1.94	1.22	0.46

Gland Size	Miami	10	-0.29 - 0.19	-0.07	-0.03	0.16	0.05
	New Port Richey	10	-0.26 - 0.20	0.08	0.04	0.14	0.04
	DeLand	7	-0.75 – 0.15	-0.14	-0.25	0.39	0.15
MBG (µg/g dry secretion)	Miami	10	44.2 – 164.10	131.50	123.40	38.83	12.28
	New Port Richey	10	5.3 – 153.50	80.75	74.30	49.74	15.73
	DeLand	7	23.8 – 188.50	131.90	113.37	52.30	19.77

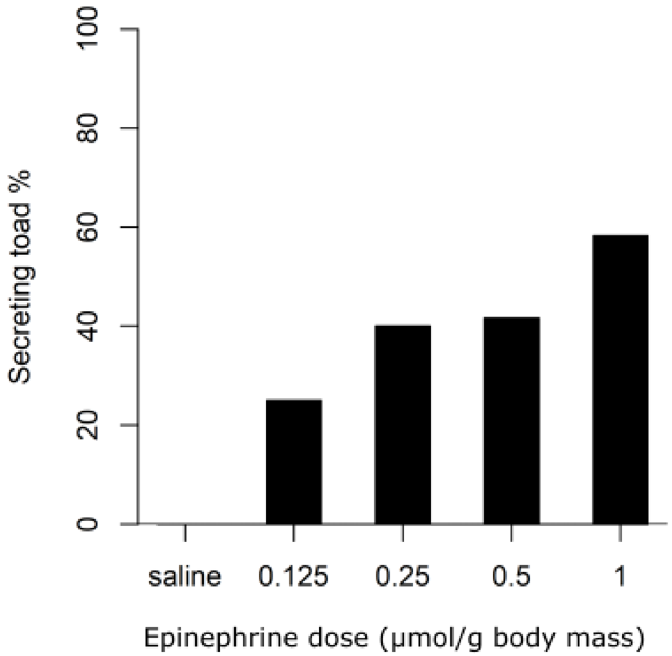


Fig 3.3 Percentages of laboratory-acclimated cane toads collected from Miami (south) (*n* = 28) and New Port Richey (north) (*n* = 30) during 2019 secreting poison following injections with increasing doses of epinephrine (µmol ((g body mass-1)). The likelihood of a toad secreting poison increased significantly with increasing dose (z55 = 2.71, *p* = .01), although there was no effect of location on this likelihood (*p* > .05)

Discussion

The cane toad range we examined in Florida extends approximately 480 km northward from the original introduction site in southern Florida. Previous studies have indicated that cane toad size ($\log_{10}$-transformed SVL) and relative parotoid gland size increases in populations near the expanding edge of the invaded range in Australia (Phillips and Shine 2005), where cane toads have established approximately 1600 km to the northwest (Brown et al., 2015) and 2200 km to the southeast (Urban et al., 2008) since their original introduction in 1935. We found that neither cane toad size nor the relationship with gland size was affected by latitude for Florida populations. Although initially our results seem contradictory to the results of Phillips and Shine (2005), the time since colonization for these populations ranges from approximately 65 years for the Homestead and Miami populations to 29 years for the northern populations such as NPR (U.S. Geological Survey 2020). Although it is unclear from our current data if the Florida populations initially showed the same pattern of increased size and gland morphology upon their initial establishment throughout the state (as time since colonization was shown to significantly affect body size and gland morphology in invasive Australian populations) it is possible that the Florida populations we sampled have now been present for long enough that increased body or parotoid gland sizes no longer afford a selective advantage (Phillips and Shine 2005).

Unlike body and parotoid gland sizes, we did observe a significant effect of latitude on BI. Studies comparing growth rates of amphibians against latitudinal or altitudinal gradients have shown that individuals from higher latitude populations show higher growth efficiency compared to those from lower latitudes (Lindgren & Laurila, 2005), perhaps as a mechanism to mitigate the effects of cooler temperatures in higher latitudes (Berven, 1982). In addition, invasive species have been postulated to escape from the pathogens and parasites of their native range upon introduction to novel habitats (Lee and Klasing 2004), with newly invading populations having a lower parasitic load due to less “carry-over” (Torchin et al., 2003). This “lag” has been observed in Australian cane toad populations undergoing expansion (Phillips et al. 2010). Although the effects of parasitic burden on BI in amphibians are variable (Moretti et al. 2014; Moretti et al. 2017), in Australia the cane toad parasite *Rhabdias pseudosphaerocephala* has been shown to exert weaker effects on infected individuals nearest to the expanding edge than in longer established populations (Phillips et al. 2010). Although parasitic burdens have not been well documented in the cane toad populations of Florida, altered burdens and other pressures associated with northward establishment could further explain the elevated BI in the northern populations.

Although we found no differences in parotoid gland size relative to body size in toads from different populations we sampled from Florida, there was an increase in the likelihood that cane toads from higher latitudes would secrete poison upon handling or disturbance. Physiologically elevated glucocorticoid levels induce phenylethanolamine-N-methyltransferase (PNMT) (an enzyme in chromaffin cells of the adrenal) to synthesize epinephrine from norepinephrine (Wurtman, 2002). Baseline corticosterone levels of the cane toads collected in 2018 were shown to increase with increasing latitude (Assis et al. 2020). Elevated corticosterone levels may have contributed to the amount of epinephrine available stored and subsequently released upon capture in toads from this study, and therefore to the likelihood of their secreting poison in the field. Mechanisms such as modified methylation patterns (Moshe Szyf et al., 2005) or altered receptor expression (Martin et al., 2017) that modulate sympathetic sensitivity are affected by early life experiences (Yao et al., 2008). As development of cane toad tadpoles occurs more slowly in cooler temperatures (Wijethunga at al. 2016), and tadpoles have been observed to readily cannibalize each other during development (Crossland and Shine 2010), prolonged development or other altered selection pressures on northern Florida populations may underlie these sympathetic differences. Additionally, Australian cane toads replenishing secretions following manual compression of the parotoid glands were shown to disperse more slowly than individuals that had not secreted poison (Blennerhassett et al., 2019). This combined with the increased number of potential predators (Punzo and Lindstrom 2001; Meshaka 2011) may contribute to range limits of cane toads in the United States.

We found no differences in MBG concentrations of cane toads from northern populations (NPR and Deland) compared to the southern Miami population. Adults have been shown to possess five primary compounds (arenobufagin, bufalin, marinobufagin, resibufagenin, and telocinobufagin) (Hayes et al., 2009b). These compounds have differing toxicities, with bufalin and telocinobufagin having higher IC_{50} values relative to the other BDs (Kamano et al., 1998). Captive-raised Australian cane toads exposed to non-lethal predator cues during larval development were shown to shift poison content toward increased amounts of bufalin compared to other compounds following metamorphosis, suggesting investment into synthesis of more lethal compounds in the presence of stronger predation cues (Hagman et al. 2009). Further research evaluating whether synthesis of more lethal compounds is higher in northern populations, complementing the higher likelihood of secretion when disturbed, would be of interest.

While the likelihood of secretion in lab-acclimated toads collected during 2019 increased with increasing doses of epinephrine, there was no difference in likelihood of secretion between northern (NPR) and southern (Miami) toad populations. The similarities in sympathetic sensitivity may have resulted from the toads being removed from the field and acclimated to the same laboratory environment prior to epinephrine injection, although altered corticosterone responses to novel stressors were still observed between toads from these populations in the study of metabolism and immune response by Gardner et al. (2020). Further studies to assess whether differences in sympathetic sensitivity between northern and southern populations are related to epinephrine release could help to elucidate the responses to injected epinephrine.

The results of this study indicate that differences in sympathetic sensitivity have arisen in cane toad populations experiencing northward dispersal in Florida. It remains unclear whether BD concentrations have been affected by the northern latitude or whether there are trade-offs between MBG and other BDs in the parotoid gland secretion. As MBG was the only BD we examined, future studies quantifying a more diverse array of BDs in cane toad secretions are needed to more accurately assess overall toxicity, and the resulting risk to native predators. Quantifying methylation patterns from populations spanning the invaded range in Florida and assessing factors leading to the differences in sympathetic sensitivity would also provide further insights, as would ecological studies assessing predation risk. Greater sampling of individuals across life history stages is also necessary to determine how the volume and composition of secretion changes ontogenetically or by region.

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Chapter 4

Chasing cane toads: assessing locomotive and behavioral changes from northward establishment in Florida

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Abstract

The cane toad (*Rhinella marina*) is an invasive species introduced to southern Florida, and populations have spread northward through the state. Populations established near expanding edges of their distributions are predicted to have higher locomotion capability relative to longer-established populations. To assess endurance, we collected cane toads from a northern (edge) and southern (core) population in Florida and placed them into a track and prodded them to hop. The number of taps, hops, and time spent hopping were recorded. Edge population individuals were less willing to move and did not travel as far in the track as those from the core population. We placed an additional subset of toads inside of a treadmill to measure endurance, where they moved until reaching exhaustion. We obtained blood samples from each individual upon completion of these trials, and once hourly for an additional three hours. Cane toads from the edge population were less willing to move in the track compared to core population individuals; however, there was no effect of locality on the distances traveled by toads in the treadmill. Although lactate levels decreased with time, there was also no effect of

locality on lactate metabolism. These results indicate behavior rather than physiological endurance in Florida cane toads has been affected by northern establishment.

Introduction

Locomotive ability affects several aspects of fitness in organisms, including the ability to secure resources (Garland Jr et al. 1990; Budick and O'Malley 2000) and avoid predators (Herrel et al. 2000; Crompton and Sellers 2007). Increases in locomotive ability may be common during biological invasions among individuals near the expanding-edge (Hughes et al. 2007; Perkins et al. 2013). Compared to organisms with static ranges, invasive species may be invading areas of high suitability that allows for their rapid spread and establishment (Travis & Dytham, 2002), with individuals dispersing into these areas potentially escaping predation (Crawley, 1986), pathogenic pressures (Torchin & Mitchell, 2004), and intraspecific competition for resources and mating (Perrin & Mazalov, 2000).

Although range expansion may offer refuge, there is also potential that individuals near these areas may experience harsher biotic (predation and competition) and abiotic (temperature) pressures (Jessop et al., 2013). In unfavorable areas, further dispersal past the range-edge may infer allele effect costs (Travis & Dytham, 2002). Due to the low numbers of individuals dispersing past this point, reproduction becomes inhibited, the likelihood of novel population establishment becomes low, and individuals displaying increased locomotive capability may be selected against (Allee, 1931; Stephens & Sutherland, 1999; Travis & Dytham, 2002).

Although initial introductions in the 1930's failed, cane toads (*Rhinella marina*) are an invasive anuran that was successfully introduced into Florida in the early 1950's (Krakauer, 1968). Since their introduction, cane toads have established populations along a south to north gradient throughout the state (Mittan and Zamudio 2019; Assis et al. 2020; Gardner et al. 2020a). Cane toads were also introduced into Australia in the 1930's, where they dispersed rapidly (Urban et al., 2007), leading to the poisoning of many naïve Australian predators (Shine, 2010a). Although increased locomotion is energetically costly (Baudinette et al., 2000)), individuals from expanding (edge) populations near the invasion front (populations established (< 1yr)) have shown increased endurance compared to longer established (core) populations (Llewelyn et al. 2010). However, this may have been due to behavioral rather than physiological changes in Australian cane toads, as indicated by the results of Tracey et al. (2012).

Compared to the longitudinal gradient of dispersal by the Australian cane toads, Florida cane toads have moved primarily along a latitudinal gradient. This has resulted in individuals from northern populations being exposed to lower mean temperatures relative to those experienced in their native range (Mittan & Zamudio, 2019). As a result, the overall rate of dispersal of Florida cane toads along their gradient—approximately 480 km over 65 years (Assis et al., 2020)—has been much slower compared to the rate in Australia (1600 km to the west and 2200 km to the south over 80 years (Urban et al. 2008; Brown et al. 2015)). Although changes to some physiological processes—such as cold tolerance, as well as immune and stress responses—have been observed along the invaded Florida range for these invasive toads (Mittan and Zamudio 2019; Assis et al. 2020; Gardner et al. 2020b), locomotion or endurance capacity has yet to be evaluated for individuals from these populations.

Our objectives were to compare northern (edge) population Florida cane toads with individuals from southern (core) populations to evaluate how locomotive capability has been affected from establishment throughout the state, as well as to assess whether Florida cane toads follow the same locomotion-capability trends as expanding-edge individuals in the Australian invasion. As Florida cane toads have spread more slowly into cooler and more challenging habitats in northern Florida—with these populations being established for ~29 years (U.S. Geological Survey 2020)—our predictions were that individuals from northern populations would show similar endurance levels as conspecifics from southern Florida, differing from the patterns of Australian cane toads.

Materials and Methods

Collection and Husbandry

We collected cane toads (*n* = 67) during May–June 2020 from Miami (25.75333°N, 80.37056°W; datum = WGS84) and New Port Richey (NPR) (28.2525°N, 82.71944°W; datum = WGS84) Florida, and transported them back to Auburn University. These localities represent the original southern-core and a northern-edge cane toad population in FL, respectively. Individuals from these populations have been used previously to compare field stress and immune responses (Assis et al., 2020) and innate immune responses (Gardner et al. 2020b). We fed toads ad libitum twice a week with crickets and maintained them in 60.96 × 46.99 × 40 cm bins at 25 ($\underline{+}$2) °C for 3 wks under a 12 h light/ dark cycle to acclimate them to laboratory conditions. We obtained morphological measurements of snout–vent lengths, masses, and hindleg lengths for all toads.

Track Locomotion

At 2100 h, during the normal active period of cane toads, we placed individual toads (*n* = 20 Miami, 24 NPR) into a 1.82 m (L) x 0.4 m (W) x 0.4 m (H) track under red lighting at 25^{o}C, and encouraged them to move by gentle tapping on the urostyle with the blunt end of a pencil. Upon reaching the ends of the track, each toad would be gently turned around and encouraged to hop back down the runway. If a toad ceased moving and remained still in place following 5 taps, the trial was concluded for that individual and the distance, time spent moving, and total number of hops taken by that individual were recorded. We recorded the total number of taps needed to encourage the toad to move until reaching exhaustion, the total number of hops taken by each toad, the total time each toad spent moving, and the total distance traveled by each toad.

Treadmill Locomotion

We placed an additional subset of cane toads (*n* = 15 Miami, 8 NPR) into a treadmill set up (similar to that used by Ward et al. (2006)). This set up consisted of a rotating clear 2000-mL beaker (12 in internal diameter) attached to swivels on a spinning rotor powered by a YH-302D power supply. We calibrated the speed of rotation by tracking the time for the spinning beaker to complete 1 rotation at 27 volts (0.044 m sec^{-}

^{1}). We chose this voltage as it yielded a rotation speed that allowed for toads to move until reaching exhaustion. The speed of rotation (m sec^{-1}) was used in conjunction with the time each toad ran in the setup to calculate the distance traveled by each toad. At 2100 h each toad was placed into the treadmill at 25^{o}C and timed with a stop-watch until reaching exhaustion, characterized by the toads remaining turned 90^{o} relative to chamber movement for 5 seconds in an attempt to cease moving. Following exhaustion, we removed each toad from the treadmill and immediately obtained a blood sample to obtain lactate levels. We assessed lactate levels using a Lactate Plus Meter (Nova Biomedical), following instructions from the manufacturer. We then placed each toad back into its respective bin to recover. This recovery period was timed, and every hour for three hours following exhaustion additional blood samples were obtained and lactate levels were recorded. Lactate levels below limits of detection were reported as the lowest detection level of 0.12 mmol L^{-1}).

Statistical Analyses

We analyzed differences in toad leg lengths of all toads collected in 2020 with multiple regression using R (version 3.6.3) (R Core Team 2019). As differences in leg morphology have been previously reported between male and female cane toads in Australia (Hudson et al., 2016), sex and location were used as factors, and $\log_{10}$ transformed SVL (toad size) was used as a covariate.

To assess differences in distances traveled by the toads, we used relative leg lengths (residuals obtained of $\log_{10}$-transformed total leg lengths compared to toad size, (RLL)) as a covariate, along with toad size. Location and sex were also used in the model as factors. A principal component analysis using the princomp function in R on the number of taps required to induce the toads to move, the total number of hops taken by the toads when moving, and the total time the toads spent moving (similar to methods used previously by Hudson et al. (2017)). The first principal component characterized 91.65% of the variation in the data and was comprised of a higher number of taps, a higher number of hops, and a longer period until the toads stopped moving. We termed this principal component “willingness to move”. We evaluated “willingness to move” for cane toads moving in the track using location and sex as factors, and RLL and $\log_{10}$ SVL as covariates.

We assessed distances traveled by cane toads in the treadmill using a similar model as used to assess distances traveled in the track, with lactate levels at the completion of the trials as an additional covariate. We assessed lactate levels of cane toads from the treadmill subset over time following the trials using a repeated measures model—using the lme function from the nlme package in R (Pinheiro et al. 2019)—using time, $\log_{10}$ transformed SVL, and RLL as covariates. Location and sex were included as factors in this model. An interaction term between time and location was initially also included; however, as this term was not significant ($P > 0.05$), it was removed from the model. Log-transformed coefficients were back-transformed, and we adjusted all resulting P values from all analyses using Bonferroni correction in R.

Results

Leg lengths of cane toads (*n* = 35 Miami (core), 32 NPR (edge)) increased by 66.8 (± 12.3) % for every 1% increase in SVL ($t_{63} = 5.40$, $P < 0.01$) (values represent mean (±) SE). Leg lengths ranged from 74.8–92.8 mm for Miami cane toads and from 53.8–94.96 mm for NPR cane toads, although locality had no effect on leg morphology ($P > 0.05$). For morphological data of cane toads collected in 2020, see Table 4.1. Leg length also did not differ between male and female toads (*n* = 25 males and 10 females from Miami, and 18 males and 14 females from NPR) (for leg morphology and distances moved for male compared to female toads, see Table 4.2).

TABLE 4.1.—Morphological data of all Florida cane toads collected in 2020.

Variable	Location	Number of individuals	Range	Median	Mean	St. dev	St.err
Mass (g)	Miami	35	63.5 – 312.0	114.6	132.71	47.94	8.10
	NPR	32	53.5 – 221.0	124.2	127.9	45.39	8.02
SVL (mm)	Miami	35	85.6 - 145.4	105.0	110.77	15.35	2.59
	NPR	32	72.60 - 129.72	103.11	104.2	12.58	2.22
Hind Leg length (mm)	Miami	35	47.8 - 92.8	76.4	75.21	10.35	1.75
	NPR	32	53.8 - 94.96	65.97	70.85	11.56	2.04
RLL	Miami	35	-0.17 - 0.09	0.014	0.00	0.06	0.01
	NPR	32	-0.11 - 0.10	-0.02	0.00	0.06	0.01

TABLE 4.2.—Morphological and locomotion data of male and female FL cane toads

Locomotion Trial	Variable	Sex	Location	Number of Individuals	Range	Median	Mean	St.Dev	St.Err
Track	Mass (g)	M	Miami	13	88.8 – 197	121.1	132.40	35.71	9.90
		M	NPR	15	74.59 - 182.40	128.4	123.88	31.73	8.19
		F	Miami	7	81.98 – 312	105.8	135.84	81.09	30.65
		F	NPR	9	86 – 221	136.9	137.69	46.70	15.56
	SVL (mm)	M	Miami	13	93.0 - 124.80	103.8	109.92	11.54	3.20
		M	NPR	15	92.30 - 120.69	104.52	105.17	8.85	2.29
		F	Miami	7	94.2 - 145.40	101.46	106.94	17.42	6.58
		F	NPR	9	92.32 - 129.72	110.2	108.90	13.01	4.34

Hind Leg Length (mm)	M	Miami	13	55.2 - 92.80	77.5	77.32	9.70	2.69
	M	NPR	15	53.80 - 83.84	66.1	67.90	8.36	2.16
	F	Miami	7	47.8 - 87.80	74.5	68.93	15.06	5.69
	F	NPR	9	56.52 - 94.96	65.5	70.07	13.16	4.39
Taps	M	Miami	13	11 - 175	85	83.92	48.37	13.42
	M	NPR	15	20 - 180	33	50.53	40.36	10.42
	F	Miami	7	21 - 124	57	72.71	38.61	14.59
	F	NPR	9	17 - 110	46	47.56	28.51	9.50
Hops	M	Miami	13	5 - 167	85	73.46	46.20	12.81
	M	NPR	15	10 - 79	25	32.87	21.68	5.60
	F	Miami	7	19 - 147	52	63.86	45.41	17.16
	F	NPR	9	8 - 79	27	38.56	29.28	9.76

	Time Moving (s)	M	Miami	13	12 - 214	109.6	98.2	56.18	15.58
		M	NPR	15	19.13 - 147.43	55.3	61.43	35.63	9.20
		F	Miami	7	22.6 - 167.0	63	81.1	49.94	18.87
		F	NPR	9	14.03 - 161.00	54.7	67.96	46.93	15.64
	Distance moved (m)	M	Miami	13	2.65 - 25.82	14.35	13.01	7.99	2.22
		M	NPR	15	2.01 - 14.71	5.87	7.15	4.24	1.09
		F	Miami	7	2.81 - 17.01	9.55	9.29	5.06	1.92
		F	NPR	9	1.27 - 21.36	4.79	7.19	6.45	2.15
Treadmill	Mass (g)								
		M	Miami	12	63.5 - 195.4	141.57	137.43	43.00	12.41
		M	NPR	3	59.50 - 107.65	106.6	91.25	27.5	15.88
		F	Miami	3	89.45 - 130.30	104	107.92	20.70	11.95
		F	NPR	5	53.5 - 214.4	173.9	144.36	77.98	34.87

SVL (mm)	M	Miami	12	85.6 - 145.3	119.25	116.14	18.72	5.404
	M	NPR	3	85.4 - 98.0	93.3	92.23	6.37	3.68
	F	Miami	3	97.6 - 107.5	100.7	101.93	5.06	2.92
	F	NPR	5	72.6 - 120.0	107.2	99.98	20.27	9.07
Hind Leg Length (mm)	M	Miami	12	65.1 - 89.5	77.6	76.73	7.48	2.16
	M	NPR	3	59.1 - 84.6	83.6	75.77	14.44	8.34
	F	Miami	3	63.9 - 81.1	78.9	74.63	9.36	5.40
	F	NPR	5	59.1 - 90.6	85.6	78.14	14.84	6.64
Time moving	M	Miami	12	96.16 - 479.65	267.61	286.59	99.30	28.66
	M	NPR	3	204.12 - 281.19	252.66	245.99	38.97	22.50

	F	Miami	3	247.47 - 261.78	261.44	256.90	8.17	4.71
	F	NPR	5	144.88 - 421.00	240.44	264.3	100.41	44.90
Distance moved (m)	M	Miami	12	4.25 - 21.20	11.83	12.66	4.39	1.27
	M	NPR	3	9.02 - 12.43	11.16	10.87	1.72	0.99
	F	Miami	3	10.94 - 11.57	11.55	11.35	0.36	0.21
	F	NPR	5	6.40 - 18.60	10.63	11.68	4.44	1.98
Lactate (mmol L-1) @ 0	M	Miami	12	2.5 - 11.4	6.4	6.73	2.53	0.73
	M	NPR	3	1.2 - 10.8	10.7	7.57	5.51	3.18
	F	Miami	3	2.9 - 7.2	4.2	4.77	2.21	1.27
	F	NPR	5	1.9 - 9.1	4.3	4.54	2.99	1.34

Lactate (mmol L-1) @ 1 hr	M	Miami	12	1.1 - 6.6	3.5	3.59	1.68	0.48
	M	NPR	3	1.6 - 5.8	4.3	3.9	2.12	1.22
	F	Miami	3	1.0 - 2.7	2.6	2.1	0.95	0.55
	F	NPR	5	0.4 - 3.2	1	1.44	1.16	0.52
Lactate (mmol L^{-1}) @ 2 hr	M	Miami	12	0.6 - 5.1	2.6	2.78	1.62	0.47
	M	NPR	3	1.1 - 3.9	2.4	2.47	1.4	0.81
	F	Miami	3	0.5 - 2.2	1	1.23	0.87	0.50
	F	NPR	5	0.12 - 1.80	0.5	0.94	0.83	0.37
Lactate (mmol L-1) @ 3 hr	M	Miami	12	0.12 - 3.60	1.65	1.78	1.08	0.31
	M	NPR	3	1.3 - 5.7	1.6	2.87	2.46	1.42

	F	Miami	3	0.4 - 1.3	0.5	0.73	0.49	0.28
	F	NPR	5	0.12 - 1.20	0.12	0.49	0.52	0.23

TABLE 4.3.—Morphological and locomotion data for subset of cane toads moving in a track.

Variable	Location	Number of individuals	Range	Median	Mean	St. dev	St.err
Mass (g)	Miami	20	81.98 - 312	111.27	133.6	53.71	12.01
	NPR	24	74.59 - 221	132.01	129.06	37.65	7.69
SVL (mm)	Miami	20	93.0 - 145.4	103.47	108.88	13.49	3.02
	NPR	24	92.30 - 129.72	105.58	106.57	10.49	2.14
Hind Leg length (mm)	Miami	20	47.8 - 92.8	75.9	74.39	12.17	2.72
	NPR	24	53.80 - 94.96	65.81	68.71	10.2	2.08

RLL	Miami	20	-0.15 - 0.11	0.02	0.01	0.07	0.01
	NPR	24	-0.10 - 0.05	-0.01	-0.01	0.04	0.01
Taps	Miami	20	11 - 175	77.50	80.00	44.48	9.95
	NPR	24	11 - 180	33.50	49.42	35.73	7.29
Hops	Miami	20	5 - 167	56.00	70.10	44.96	10.05
	NPR	24	8 - 79	26.00	35.00	24.33	4.50
Time moving (s)	Miami	20	12 - 214	95.5	92.22	53.4	11.94
	NPR	24	14.03 - 161	55	63.88	39.36	8.03
Distance moved (m)	Miami	20	2.65 - 25.82	10.44	11.71	7.19	1.61
	NPR	24	1.27 - 21.36	5.75	7.17	5.04	1.03

Distances traveled by cane toads in the track ranged from 2.65–25.82 m for Miami cane toads, and from 1.27–21.36 m for NPR cane toads (for full track data see Table 4.3). Individuals from Miami traveled 5.31 (± 1.87) m further than toads from NPR (t_{39} = 2.85, *P* = 0.03) (Fig. 4.1); with NPR toads being less willing to move in the track compared to Miami cane toads (t_{39} = -3.06, *P* = 0.02); however, there was no effect of sex (*n* = 13 males and 7 females from Miami, and 15 males and 9 females from NPR), SVL, or RLL on the distance traveled by cane toads in the track or their willingness to move (*P* > 0.05).

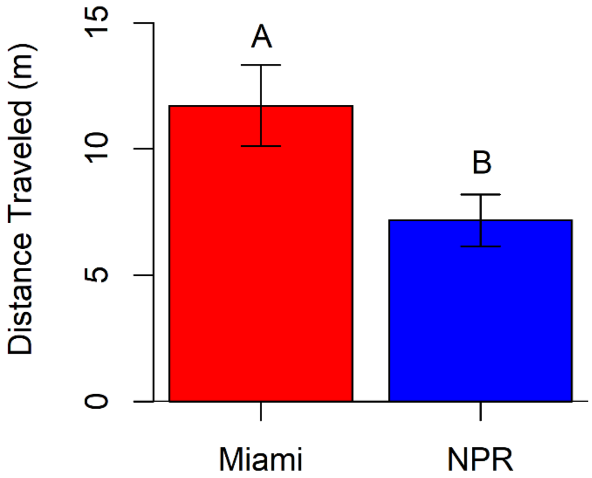

FIG 4.1 Distance traveled in a track by laboratory-acclimated cane toads from a southern core (Miami (*n* = 20)) and northern edge (New Port Richey (NPR) (*n* = 24)) Florida population. Cane toads from both populations were encouraged to move with gentle prodding. Cane toads from the southern population were more willing to move compared to individuals from the northern population, with differing letters indicating significant differences ($P < 0.05$).

Distances traveled by cane toads in the treadmill ranged from 4.25–21.20 m for Miami cane toads, and 6.40–18.60 m for NPR individuals (for full treadmill data see Table 4.4). Distances did not differ between the populations, and were not affected by lactate levels, SVL, RLL, or sex (n = 12 males and 3 females from Miami, and 3 males and 5 females from NPR) ($P < 0.05$) (Fig. 4.2). Lactate levels ranged from 2.5–11.4 mmol L^{-1} for Miami cane toads and from 1.9–10.8 mmol L^{-1} for NPR cane toads at exhaustion. Although these levels decreased by 1.48 (± 0.13) mmol L^{-1} h^{-1} ($t_{68} = -10.94$, $P = 0.03$) in recovering toads following locomotion in the setup (Fig. 4.3), there was no difference in lactate levels between the populations ($P > 0.05$). These levels were also not affected by RLL, SVL, or sex ($P > 0.05$).

TABLE 4.4.—Morphological and lactate data for subset of cane toads moving in a treadmill.

Variable	Location	Number of individuals	Range	Median	Mean	St. dev	St.err
Mass (g)	Miami	15	63.5 - 195.4	130.3	131.53	40.79	10.53
	NPR	8	53.5 - 214. 4	107.13	124.44	66.68	23.57
SVL (mm)	Miami	15	85.6 - 145.3	110.9	113.3	17.71	4.57
	NPR	8	72.6 - 120	95.65	97.08	16.2	5.73
Hind Leg length (mm)	Miami	15	63.9 - 89.5	78.8	76.31	7.56	1.95
	NPR	8	59.1 - 90.6	84.1	77.25	13.68	4.84
RLL	Miami	15	-0.06 - 0.05	-0.015	-0.01	0.03	0.01

	NPR	8	-0.06 - 0.07	0.042	0.02	0.06	0.02
Time moving (s)	Miami	15	96.16 - 479.65	261.44	280.65	88.92	22.96
	NPR	8	144.88 – 421.00	246.55	257.43	79.28	28.03
Distance moved (m)	Miami	15	4.25 - 21.20	11.55	12.4	3.93	1.01
	NPR	8	6.40 - 18.60	10.9	11.38	3.5	1.24
Lactate (mmol L^{-1}) @ 0	Miami	15	2.5 - 11.4	6.3	6.34	2.53	0.65
	NPR	8	1.9 - 10.8	4.9	5.86	3.82	1.35
Lactate (mmol L^{-1}) @ 1h	Miami	15	1.0 - 6.6	2.7	3.29	1.65	0.43

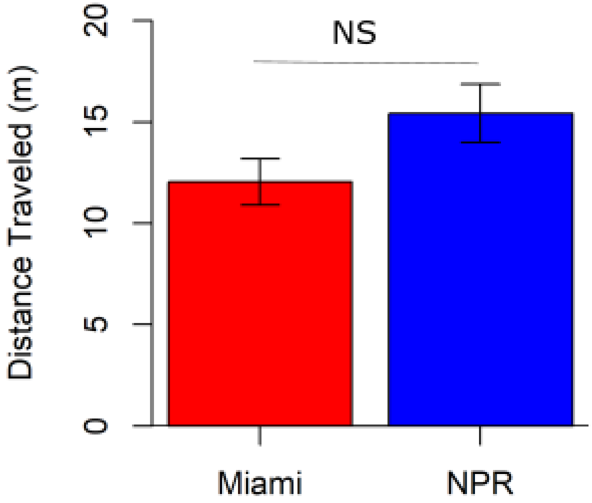

FIG.4.2.—Distance traveled by FL cane toads collected from a southern core (Miami, *n* = 15) and northern edge (New Port Richey (NPR), *n* = 8) population in a treadmill-like device. NS indicates distances traveled were not significant based on locality (*P* > 0.05).

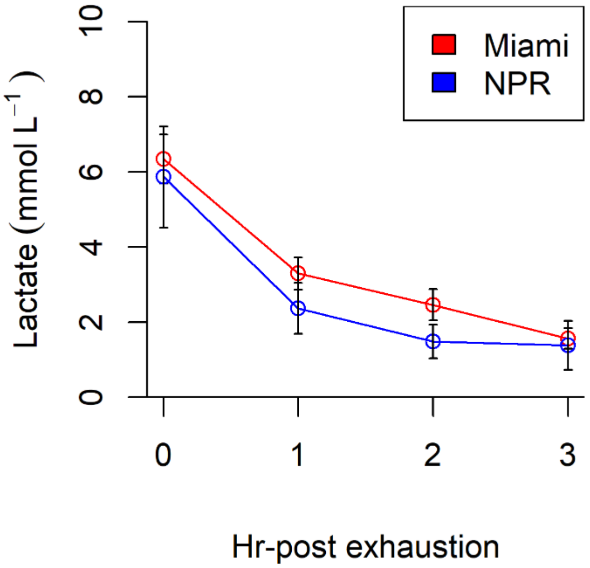

FIG.4.3.—Lactate levels of southern core (Miami, *n* = 15) and northern edge (NPR, *n* = 8) cane toads obtained over a 3-h time period that began immediately upon conclusion of locomotion trials in a treadmill-like setup. Although lactate levels decreased over time for all individuals, changes in lactate did not differ based on location ($P > 0.05$).

Discussion

Longer relative leg lengths in amphibians affects locomotor performance (Zug, 1972). Near the expanding edge of their distribution in 2010, Australian cane toads had longer legs compared to individuals from longer established core populations (Llewelyn et al., 2010). Larger toads were also faster in a track, although there were no differences based on location, and RLL did not significantly affect speed. In comparison, although leg lengths of cane toads in our study increased with increasing toad size, we observed no differences in the leg morphology or endurance of northern edge compared to southern core Florida population cane toads. This is similar to parotoid gland morphology, which also did not differ among the populations in Florida (Gardner et al. 2020a). There were also no differences in these parameters between male and female cane toads used in this study.

Recently, Rubio et al. (2020) indicated that cane toads from Florida populations near those sampled in our study showed a similar trend as Australian cane toads, possessing longer relative legs lengths when initially dispersing and establishing throughout the state. However, Rubio et al. (2020) also found that proportions of longer legged individuals in these populations decreased in years following establishment, similar to results from the study by Hudson et al. (2016) regarding Australian cane toads. Proportions of longer legged individuals were observed to increase again in expanding Australian populations upon further dispersal (Hudson et al. 2016); however, as we observed no differences in RLL between our sampled north and south populations and the NPR Florida population has now been established for approximately 29 years (U. S.

Geological Survey 2020), the results of Rubio et al. (2020) and our current results may indicate that proportions of longer legged individuals in northern Florida populations are now reduced, similar to older Australian populations (Benjamin L. Phillips et al., 2006a). This may also indicate that the initial selective pressures that favored longer leg lengths—conferring higher locomotion capability—in dispersing Florida cane toads may have diminished as populations became established in northern areas with lower mean temperatures, playing a role in limiting further dispersal.

Compared to the 11.7 (± 1.6) m and 7.2 (± 1.0) m traveled by Miami and NPR cane toads in our track, respectively, similarly prodded cane toads in the study by Llewelyn et al. (2010) traveled greater distances in a track ranging from approximately 50 m for individuals from longer-established populations and 220 m for individuals from invasion-front populations. Australian cane toads from both invasive-front and longer established populations also showed higher endurance and locomotor performance compared to individuals collected from both their native range in Brazil and invasive populations in Hawaii (Kosmala et al. 2017). This likely indicates that differing selective pressures in Australia favor increased movement in cane toads compared to other localities. Regarding the Florida populations we sampled in our study, individuals from NPR showed less “willingness” to move in the track, evidenced by our 1st principal component and the lower distance they traveled compared to Miami cane toads. As increased dispersal has been correlated with “boldness” behavior (Cote et al. 2010; Myles-Gonzalez et al. 2015), cane toads from NPR being less willing to move, i.e. being less “bold”, could offer additional support that further dispersal in Florida has lessened.

Although increased locomotive ability may be more favorable in dispersing individuals establishing new populations (Travis and Dytham 2002), arrival into territories with increased predation pressures may favor decreased movement (Siepielski et al., 2016). Remaining motionless is an initial predation defense for amphibians (Caldwell et al. 1980; Feder 1983; Toledo et al. 2011), offering protection against predators such as birds or snakes (Brodie Jr, 1977; Marchisin & Anderson, 1978). If remaining motionless fails, the primary defense to predation attempts by cane toads is to secrete poison, which is located primarily in the parotoid glands of cane toads where the toxic compounds are synthesized (Hayes et al. 2009; Mailho-Fontana et al. 2014). Increased toxin synthesis by cane toads is energetically costly, as evidenced by decreased liver and gonadal sizes in Australian individuals possessing larger toxin stores and decreased growth rates in toads replenishing stores following manual compression of their glands (Blennerhassett et al., 2019). Locomotion has also been shown to be energetically costly for these toads, with sustained movement of cane toads on a treadmill incurring an energetic cost of 21.68 J kg^{-1} m^{-1} in the study by Baudinette et al. (2000), assuming that 1 mL of O_2 consumed was equivalent to 20.11 J in non-postabsorptive cane toads. With the average mass of cane toads from both NPR and Miami in the current study being approximately 130 g, and the average distances traveled being 7.17 m and 11.71 m for individuals from these populations, respectively, this would roughly translate to 20 compared to 33 joules of energy or 4.8 compared to 7.9 calories consumed for individuals from NPR and Miami populations traveling in our track (a 1.65-fold difference in energy consumed, respectively).

Although the underlying causes are unclear, a previous study by Assis et al. (2020) showed that cane toads from established northern populations in FL have higher baseline corticosterone levels compared to southern population cane toads. Additionally, Gardner et al. (2020a) showed that the likelihood of cane toads to secrete poison upon handling or disturbance increases with population latitude in Florida. As a few native Florida predators—such as snake species, opossums, and birds (Meshaka Jr, 2011)—have been observed consuming adult toads, these results may be indicative of higher predation pressures in these areas. It is possible that individuals from NPR and other northern Florida populations may favor reduced movement as a result, or from the associated increased energetic costs of replenishing toxin stores compared to cane toads from more southern populations.

As there were no differences in the distances traveled by the toads from NPR (edge) compared to Miami (core) when placed in the treadmill, it is unsurprising that lactate levels were not affected by locality. This was also the case for cane toads in the study by Tracey et al. (2012), which indicated little change in underlying locomotion physiology has occurred among toads from the invaded range in Australia. Amphibian locomotor performance is affected by temperatures and hydration levels (Preest et al. 1992; Walvoord 2003; Kosmala et al. 2017). Although we observed no effect of locality on cane toad endurance, we did not compare endurance levels at differing temperatures, nor did we compare whether toads from the different localities differ in resistance to desiccation. Mittan and Zamudio (2019) have shown that cane toads from northern populations have lower CTmin values. It is possible that increases in cold tolerance, in addition to increasing mean environmental temperature, could confer further dispersal capability to cane toads in northern Florida with time. Monitoring of these edge populations to compare locomotor capabilities of individuals in the event of further northward dispersal, as well as under altered hydration and temperature regimes is of interest in future studies.

Our results indicate that behavioral changes have occurred in FL cane toads as a result of their northern establishment, which affect the likelihood of them fleeing with disturbance. Exposure to early-life or chronic stress has been correlated with altered expression of various HPA-axis receptors (Szyf et al. 2005,2007; Turecki and Meaney 2016), which affects behavioral and physiological responses to stressors occurring later in life (Denver, 2009). As such, additional studies comparing expression of these receptors in toads from differing populations could be beneficial for understanding the underlying physiological mechanisms that result in decreases in northern cane toad movement, as well as ecological studies that determine if higher predation pressures in these areas also contribute to these changes.

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Chapter 5

Discussion and Conclusion

Our predictions were that northern-edge population Florida cane toads would show attenuated immune responses, increased parotoid gland morphology and secretion likelihood, and similar endurance and leg morphology compared to southern-edge toads. The results we observed indicate that with the exception of northern Florida cane toads having larger parotoid glands relative to their body sizes, our predictions were supported by the data we obtained. Additionally, a consistent factor we observed among our data was the differing sensitivity patterns that cane toads from northern Florida populations displayed compared to those from the southern populations.

Amphibians and other ectotherms from higher latitude populations or altitudes have shown tendencies to have slower growth, larger adult sizes, and fewer numbers of offspring (Morrison & Hero, 2003) compared to those from lower latitudes and altitude localities. Indeed, cane toads near southern areas of the range in Australia have been observed growing slower and having larger masses upon metamorphosis compared to warmer population individuals (Wijethunga et al., 2016). This may play a role in the increased body conditions we observed among northern Florida cane toads compared to those from southern Florida populations (discussed in Chapter 3).

Northern population cane toads in Florida have shown increased tolerance to cooler temperatures (Mittan & Zamudio, 2019), enabling individuals from these populations to survive. However, cane toads from the Tampa area in Florida (just south of the NPR population sampled in our research) did not demonstrate increased locomotive ability when subjected to cooler temperatures. Mittan and Zamudio (2019) predicted that as cane toads lack freeze tolerance mechanisms observed in other amphibians (Storey & Storey, 1986), and northern areas of Florida occasionally experience freezing temperatures, further dispersal and population expansion into more temperate areas of northern Florida are likely limited. Based on the work and predictions of Mittan and Zamudio, our own data that indicated leg morphology did not differ between our sampled north and south populations, and the study by Phillips et al. (2006) which indicated that cane toads from populations that are no longer expanding do not display increased leg lengths, our hypothesis is that the Florida cane toads as of now are limited to their current range in Florida.

Regarding the implications suggested by our data that cane toads living in northern Florida experience more stressful conditions (i.e. environmentally lower temperature minima), genetic analysis using single nucleotide morphisms (SNPs) of invasive cane toad populations in Australia revealed that the population sub-structuring, or FST levels, among the Australian populations is highest when comparing core population individuals to those that have spread into the cooler climates in the southeastern areas in Australia (Trumbo et al. 2016). Gene flow from the core populations to those in these areas was restricted, giving indication that lower minima temperatures are a likely stressor that may limit genetic diversity and dispersal of these invasive toads (Trumbo et al. 2016). The increased GC levels of the cane toads in northern areas of FL may therefore stem in part from developing, maturing, surviving, and reproducing in the more temperate areas in central and northern Florida (Krakauer, 1968). As mentioned in chapter 3, elevated glucocorticoid levels also increase epinephrine synthesis (Wurtman, 2002), which likely resulted in the increased poison secretion likelihood of the northern cane toads we observed.

Higher latitude populations generally experience shorter breeding seasons, which can increase inter-male competition (Eikenaar et al., 2012). The increased energetic demands on amphibians breeding in lower temperature habitats while also enduring increased competition may lead to elevated baseline corticosterone levels in adults (Hau et al., 2010). Juvenile amphibians, including cane toads, have been observed to have delayed hatching at cooler temperatures (Burraco et al., 2020; Wijethunga et al., 2016). Under fluctuating temperatures, amphibian larvae that initially showed delayed hatching have displayed compensatory growth patterns (Orizaola et al., 2010; Hector et al., 2012), rapidly catching up in size to conspecifics raised in warmer temperatures. This was shown in *Rana temporaria* tadpoles, although this was associated with increased glucocorticoid levels as well as increased antioxidant enzyme activity (Burraco et al., 2020).

Epigenetic mechanisms such as DNA methylation or modification to histone structures may alter the phenotypes observed among individuals from populations that are lacking wider ranges of genetic diversity (Bossdorf et al., 2008). Invasive species typically experience founder effects, where genetic diversity of the introduced populations is limited based on the few numbers of individuals available in the introduction (Allendorf & Lundquist, 2003). This reduces available genetic diversity among introduced populations, which is thought to be needed for these individuals to survive, establish, and spread through the new environment (Blackburn et al., 2015). In many cases however, populations of introduced species have been observed thriving in novel environments despite lower effective population sizes (Ardura et al., 2017; Marin et al., 2020). It is thought that increased plasticity from epigenetic mechanisms such as methylation, non-coding RNA, and transposable elements preexisting in the genomes of these species may play a role in allowing these organisms to invade (Marin et al., 2020).

Studies showing that invasive species show altered epigenetic patterns are becoming more common (Schrey et al., 2012). For example, invasive pygmy mussels in Europe from recently established populations were observed with lowered methylation patterns compared to older populations (Ardura et al., 2017), which were predicted to offer increased plasticity to enable establishment and compensate for reduced genetic diversity. Other examples include increased methylation of a heat shock protein (hsp90) and an ion transporter gene were found in the invasive ascidian *Ciona robusta*, which conferred increased salinity and temperature tolerance (Pu & Zhan, 2017), and increased methylation patterns in the invasive mosquito *Aedes albopictus* that increased egg cold hardiness (although the exact genes responsible were not evaluated) (Kreß et al., 2017).

It has been suggested that the transposable elements that normally are present in the genomes of introduced species become active upon the increased novel stress of living in and colonizing novel habitats (Barrón et al., 2014), potentially as a result of hypomethylation that has been shown to occur upon the initial expansion phases of biological invasions (Stapley et al., 2015). This increase in transposable element activity can lead to altered gene expression patterns and novel mutations, which may increase variation in expanding populations and enable individuals to persist in environments that would otherwise have been deleterious (Vieira & Biémont, 2004). In addition to the increased methylation patterns mentioned previously for the invasive mosquito *Aedes albopictus*, higher numbers of transposon insertions among individuals from invasive European populations were observed, which were postulated to occur due to establishment in more temperate areas (Goubert et al., 2017).

Regarding the effects altered methylation patterns may have on stress physiology, prolonged exposure to high glucocorticoid levels early in development influence DNA methylation patterns (M. Szyf et al., 2007; Moshe Szyf et al., 2005; Turecki & Meaney, 2016), and affect behavioral and physiological responses throughout life (Denver, 2009). Studies with other amphibians have shown that elevated CORT levels early in development alter growth (Hayes 1997; Denver et al., 2002). Developing *Xenopus laevis* tadpoles with restricted food availability (that were also given exogenous corticosterone) had different growth rates (Hu et al., 2008). In addition, increased methylation of glucocorticoid receptor genes leads to decreased receptor expression, leading to decreased efficiency of the negative feedback loop associated with organisms mounting responses to stressors (Bodegom et al., 2017). This was also observed in *Xenopus laevis* tadpoles, which showed decreased responsiveness from GCs in GC-reactive regions of their brains (Hu et al., 2008), and also showed decreased sensitivity to a physical stressor later in life (Yao et al., 2008). Weaver et al. (2004) showed a promoter region within the rat GC receptor gene that becomes differentially methylated depending on early life stressors, a homologue of which has also been found in amphibians (Hu et al. 2008). In addition to altered methylation patterns in the hypothalamus and pituitary glands, altered patterns have also been observed in the prefrontal cortex and amygdala (Urb et al., 2019; Hegde et al., 2020), as well as around regions near serotonin receptor genes (Alexander et al., 2014), all of which may also contribute to differing reactivity to novel stressors. Reduced methylation of the CRH promoter region can also occur, leading to increased expression of CRH that leads to further increases in baseline GC levels (Chen et al., 2012; Xu et al., 2014).

Individuals that display stationary behaviors when experiencing novel stressors, as well as having chronically elevated GC levels while showing decreased GC responsiveness are commonly referred to as having “reactive” personality types (Careau et al., 2008) while others that have higher levels of “exploratory” or active behavior when subjected to novel conditions coupled with increased GC responsiveness are referred to as “proactive”. Proactive individuals show trends of increased investment in innate immune responses (Zylberberg et al., 2014).

Much attention has been paid to the EICA hypothesis mentioned in chapters 1 and 2 (Cornet et al., 2016), which predicts that introduced species are able to become invasive due to increased competitive ability as a result of having to mount weaker immune responses (Lee & Klasing, 2004). Indeed, many of these studies have shown that invasive species have attenuated immune responses compared to native species (Lee et al., 2005; Cornet et al., 2010; Marin et al., 2020), and variation among edge and core introduced populations has also been documented (Llewellyn et al., 2012; Brown et al., 2015; Quéméré et al., 2015)(. However, many of these studies did not take into account the effects that glucocorticoids have on modulating these responses, and how these levels may be affected from dispersal and establishment throughout the novel environment. As indicated by Stapley et al. (2015), rather than comparing immune investment of invasive species as a measure of their invasive potential, it may be more beneficial to instead compare the levels of genetic diversity that exist among these populations with epigenetic patterns such as methylation and TE activity. Although we do not currently have population genetic data for the Florida cane toad populations, our hypothesis is that similar to the southern Australian populations, Florida cane toads likely experienced founder effects from their initial establishment (Krakauer, 1968), and genetic diversity has likely become further reduced in the northern Florida populations.

Personality differences may also exist among ectotherms living at differing thermal regimes in their range (Michelangeli et al., 2018). There is evidence of ectotherms living in warmer environments having faster growth, higher endurance levels, and increased exploratory behaviors compared to individuals from more temperate populations (Brodie and Russel 1999; Goulet et al., 2017), which has been postulated to be due to the increased metabolic demands of living in areas with higher mean temperatures. For example, delicate skinks (*Lampropholis delicata*) from populations experiencing warmer mean temperatures were more active, explorative, and had higher sprint speeds compared to cooler conspecifics (Merritt et al., 2013). These patterns have also been observed in fish (Nile Tilapia) (Cerqueira et al., 2016), and mountain log skinks (*Pseudemoia entrecasteauxii*) (Stapley, 2006).

Our observation based on our results is that cane toads from the northern populations tend to show “reactive” personalities, while those from the southern populations are more “proactive”, likely as a result of the northern populations experiencing lower thermal regimes. This is indicated by our results of 1. the differing CORT responses displayed by the NPR and Miami population cane toads over time in the metabolic study, 2. the toads from Miami being more active and attempting to escape compared with NPR individuals who remained stationary when receiving injections in Chapter 2 (personal observation). 3. NPR cane toads receiving LPS injections not showing increased metabolic rates compared to baseline rates while those from Miami did, 4. Northern population cane toads showing increased likelihoods of secreting poison compared to toads from the southern populations, and 5. NPR cane toads being less willing to move in our track compared to Miami cane toads. The field study by Assis et al. (2020) in which cane toads from south and north Florida populations were subjected to a restraint challenge and their baseline and post-restraint corticosterone levels were compared further indicates the differing personality and coping styles of the northern Florida cane toads.

Overall, we hypothesize that the differences we observed between northern and southern Florida cane toads in our data are a result of altered epigenetic patterns that have occurred in individuals from the northern populations as a consequence of the differing conditions they experience. Therefore, further work obtaining genetic data for the Florida cane toad populations to test this hypothesis while also comparing genetic diversity with methylation and other epigenetic patterns in the Florida cane toads would allow for us to assess if these toads were able to establish and persist in northern Florida as a result of increased plasticity in the face of decreased genetic diversity. Further research assessing methylation patterns of GC receptor regions among the invasive cane toad populations in Florida could therefore reveal altered methylation patterns as being the mechanistic cause of the differential responses we observed. These modifications may last several generations, leading to the behavioral and physiological tradeoffs associated with differential stress responses to persist within the populations experiencing them (Bossdorf et al. 2008; Franklin et al. 2010; Tureki and Meaney 2016).

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